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Marine Biological Laboratory

WOODS HOLE, MASS.

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Vol. LII *March, 1927* *No. 3*

BIOLOGICAL BULLETIN

THE EFFECT OF LACK OF OXYGEN ON SEA URCHIN EGGS.

ETHEL BROWNE HARVEY.

It has long been known that fertilized sea urchin eggs may live for some time in the absence of oxygen without division, and divide on readmission of oxygen (Loeb, '95). Since the rôle of oxygen is of fundamental importance in the life of the cell, I thought it might be of interest to make a study of the changes that take place in individual eggs during lack of oxygen at different stages of development, with special reference to the mitotic figure. The opportunity to procure excellent material for this study was given at the Naples Laboratory, where the perfectly transparent eggs of *Echinus microtuberculatus* and *Strongylocentrotus lividus* may be procured during the entire year. It is with great pleasure that I thank Dr. Dohrn for his kindness and courtesy while working in the Stazione Zoologica, and also the Association to aid Scientific Research by Women for the use of their room.

The observations were made on eggs in a hanging drop in a modified Engelmann's chamber to which pure hydrogen [1] was admitted. The eggs were entangled in threads of platinized asbestos to absorb the last traces of oxygen, and they were stained in methylene blue in order to tell when the oxygen was completely gone. Under the conditions of the experiment, it was found that it took about twenty minutes for the oxygen to be entirely used up as indicated by the colorless condition of the originally blue stained eggs. The eggs of *Echinus microtuberculatus* and *Strongylocentrotus lividus* gave practically the

[1] The hydrogen was prepared in a Kipp generator from zinc and sulphuric acid washed with alkaline permanganate and passed over platinized asbestos heated to redness in a quartz tube.

same results, and were equally good for observation, the eggs of *Echinus* being a little larger.

When the eggs are subjected to lack of oxygen immediately after fertilization (Fig. 1), the sperm aster forms and the two pronuclei approach just as under normal conditions, there being still sufficient oxygen for normal development. After about twenty minutes, however, the process is arrested owing to lack of oxygen. In some cases this takes place when the two pronuclei are still a little distance apart (Fig. 2). If kept in absence of oxygen, the sperm aster gradually fades out and the cell appears just as it did on fertilization (Fig. 3). This usually occurs in 40 to 60 minutes after admission of hydrogen to the chamber (room temperature 16°–18°). In most cases the female pronucleus is still visible even after two or three hours in hydrogen, but in some cells it cannot be discerned. In most of my experiments at this stage, the cells became irregular in outline after about forty minutes in hydrogen, sometimes earlier, and much crinkled, showing decidedly light and dark areas of protoplasm, and they remained in this condition, gradually increasing in intensity for two or three hours (Figs. 4 and 5). I am inclined to think that this is due, however, to other conditions than lack of oxygen since in two experiments most of the eggs remained uncrinkled and quite normal in appearance, and sometimes eggs under apparently normal conditions show the same peculiarities. They are somewhat similar to eggs kept in hypertonic sea water, although it cannot be due here to withdrawal of water since the same eggs in the same chamber later became smooth again.

When oxygen is admitted, the sperm aster reappears, in the same position where it disappeared, in the course of half an hour in the clear uncrenulated eggs, and development goes on in quite a normal fashion though all the stages are of course delayed. In the crenulated eggs, the dense protoplasm obscures the nuclear phenomena, and usually the mitotic figure is in the early metaphase before asters or nuclei are discernible (Fig. 6). Even though much crinkled and irregular, these eggs straighten out, become regular in outline and normal in appearance. The only abnormality observed in the development of the eggs

which were subjected to hydrogen immediately after fertilization is in the first division plane sometimes coming in so as to divide the egg into two slightly unequal cells. In most experiments, the eggs developed into normal swimming larvæ next morning.

Eggs subjected to hydrogen at any later stage of development up to the formation of the metaphase spindle behave similarly to those described, and I have tried every stage. The astral rays gradually disappear under hydrogen, leaving the egg quite homogeneous; the eggs may become crenulated and irregular especially in the early stages. But when oxygen is admitted, the astral rays reappear and the eggs divide and develop normally except for the occasional unequal division of the first two cells. When the eggs are perfectly clear, the mitotic figure always reappears when oxygen is admitted *in exactly the same phase and position* in which it disappeared when oxygen was taken away.

When the eggs are kept without oxygen while in the full metaphase, in some cases the fibers become indistinct in about half an hour, but usually they remain distinct for an hour or more; in some cases the astral fibers have persisted longer than two hours. After the fibers disappear, the eggs become almost homogeneous, with perhaps a few darker areas (Fig. 7). When the eggs, stained in methylene blue are becoming decolorized by lack of oxygen, the blue remains last in the granules among the astral rays. This region is also a little more blue in the normal eggs stained in methylene blue than elsewhere, and is especially so in subsequent cleavages. In the very granular eggs of *Sphærechinus granularis*, the region around the spindle and asters is very blue while the periphery may be quite colorless.

When oxygen is admitted to the eggs which have been in a hydrogen atmosphere during the metaphase, the asters may reappear in the course of an hour in the same place where they disappeared (Fig. 8). In many cases, however, the mitotic figure assumes a peculiar appearance, a decided white streak running between the two asters and the astropheres are elongated in one axis (Figs. 19, 24). The white streak gradually disappears (Figs. 20, 21, 25, 26).

The eggs which have been subjected to lack of oxygen during the metaphase pursue the most peculiar development on re-

admission of air. Instead of dividing into two, frequently several asters appear (Figs. 9, 21, 28) and the egg divides quite irregularly. Sometimes one fairly large cell divides off (Fig. 10), sometimes several large lobes start to constrict off (Fig. 22), sometimes many very small fragments pinch off (Figs. 27, 28, 32, 33). Sometimes the constrictions complete themselves and form cells, sometimes they again fuse with the main cell body. The following stages are likewise irregular, the cells seeming to attempt all sorts of methods of righting themselves by trying first one plane and then another, meanwhile obliterating a plane already in existence.

These eggs seemed so abnormal in appearance that I at first threw them aside thinking that they had been harmed by some experimental conditions and that they were about to die. But much to my surprise, I found that many living larvæ were present in some of the chambers one morning. I have since watched many individual eggs pass through these most amazing stages, assuming the most bizarre appearance, cells of all sizes pinching off and again fusing in the most irregular fashion. But these same eggs succeed in righting themselves, and finally give rise to a number of equal cells, and go on with a regular cleavage resulting in normal blastulæ. Stages in the development of four such eggs are given in Figs. 7–18, 19–23, 24–31, 32–35. Even a very short exposure to the absence of oxygen (5 minutes) is sufficient to call forth these peculiarities when air is readmitted. A long exposure at any other stage previous to the full metaphase does not give this result.

If the cell has elongated preparatory to division after being in the chamber for about twenty minutes, that is, during absence of oxygen, if kept longer in hydrogen, it will again round out and if a division plane has started as it sometimes does, it will again become resorbed (Figs. 36–38, 39–41). In many cases a division plane has started to come in after the egg has been in the chamber 30 minutes, more than time for all the oxygen to have gone, and after the blue color of the methylene blue has gone but before the mitotic figure has faded out, but this is resorbed if kept in hydrogen. On readmission of oxygen, these eggs proceed with the same sort of irregular cleavages as those in the full metaphase, but result finally in normal blastulæ.

When the division plane has completely formed before total absence of oxygen, it remains, and when oxygen is admitted the cleavage goes on regularly, the egg dividing into two equal cells, then four, etc. The division planes which come in during a scarcity but not total absence of oxygen (*i.e.*, about 15 min. of hydrogen) are often somewhat crinkled and irregular (Fig. 42).

If the egg is subjected to lack of oxygen just before the second cleavage, when air is admitted, the same peculiar irregular divisions are formed as when exposed to hydrogen during the first metaphase (*i.e.*, just before the first cleavage), followed by fusion and irregular divisions, but resulting in normal blastulæ. If subjected to lack of oxygen at any stage between the completion of the first cleavage and the full metaphase of the second, the cleavage goes on normally when air is admitted.

These experiments show that sea urchin eggs may be deprived of oxygen at any stage, from just before the union of the two pronuclei until the completion of the first cleavage, and will remain in whatever stage they may be in, without further development, if kept without oxygen. Astral rays, whether of the sperm aster or the first cleavage asters at any stage, gradually disappear until the egg becomes quite homogeneous. The first cleavage furrow may start to come in during lack of oxygen, but is later resorbed. When oxygen is again admitted, the astral rays reappear in exactly the same position where they disappeared, the mitotic figure reappears in the same phase, and development proceeds. The reappearing mitotic figure and subsequent cleavage is perfectly normal at any stage except in those eggs which were kept without oxygen at the metaphase or a little later, when the reappearing mitotic figure is peculiar and the cleavages are quite irregular. Even the very abnormal looking eggs resulting from the irregular cleavages, however, right themselves and give rise to perfectly normal larvæ.

In Loeb's ('95) experiments, the eggs were exposed to lack of oxygen at only one stage, *i.e.*, soon after fertilization, and his results are quite in accord with mine for this stage. Demoor ('95) thought that in the cells of *Tradescantia*, one could make a distinction between protoplasmic life and nuclear life in their relation to oxygen, the latter going on in spite of lack of oxygen

resulting in a continuation of mitotic division, while the protoplasmic life was arrested, no streaming of protoplasm taking place and no cell divisions. Such a distinction can certainly not be made in the case of the sea urchin egg, for practically all manifestations of life are inhibited by lack of oxygen, both nuclear and protoplasmic.

The fading out of the astral fibers and disappearance of the mitotic figure which resulted from exposure to lack of oxygen, are of interest in comparison with the early experiments of the Hertwigs ('87 and '90) with chloral hydrate, quinine and cold, and those of Wilson ('01) on ether. Perhaps the disappearance is even more complete in the case of absence of oxygen, for all traces of fibers, astral rays and astrospheres may be lost.

As indicated above, the lack of oxygen had practically no effect on subsequent development during any of the earlier stages of mitotic division. The astral rays and spindles come back in the same place and condition when oxygen is readmitted as they were before oxygen was taken away. It is only when oxygen is taken away during the full metaphase or a little after that the reappearing mitotic figure is different and the subsequent cleavages are aberrant, although even here normal larvæ are produced. This is contradictory to the results of E. P. Lyon ('02), who found that sea urchin eggs were most susceptible to lack of oxygen and to cold 10–15 minutes after fertilization. Although in my experiments, eggs were not in total absence of oxygen till 15–20 minutes after fertilization, they were at a lower temperature (*i.e.*, 16°–18° as opposed to 20° of Lyon) so that most likely his susceptible period was covered. The period that was most susceptible in my experiments was the metaphase and slightly later, a period which Lyon ('02, '04) found most resistant to lack of oxygen and cold but most susceptible to heat and most productive of CO_2. However, the criterion of susceptibility was different in his experiments and mine, his being the total number of survivors and mine being the microscopic appearance of individual eggs. The fate of the larvæ was quite precarious in my experiments, some lots of eggs going well and others under apparently identical conditions not surviving the following morning. When conditions are

favorable, normal larvæ are produced after readmission of oxygen no matter at what stage in mitosis the eggs are subjected to lack of oxygen.

It might be of interest to compare briefly the results on oxygen consumption of the sea urchin eggs with my results on lack of oxygen. Warburg ('15) found that 10 minutes after fertilization, the oxygen consumption in *Strongylocentrotus* was six times that of the unfertilized egg and in the sixth hour was twelve times. Shearer ('22) by means of a special type of Barcroft manometer found that the oxygen consumption of *Echinus* eggs was eighty times as great during the first minute after fertilization as during the minute just before fertilization. The tremendous increase in oxygen consumption occurs at the time that the sperm comes in contact with the egg, before entrance. My experiments have not included this early stage owing to difficulties in technique. But it is interesting that Shearer obtained no special increase in oxygen consumption at the time of the union of the pronuclei, a stage which I found unaffected by lack of oxygen, whereas he did obtain an increase at the time of first cleavage (45–50 min. after fertilization), this corresponding roughly to the period just before first cleavage in my experiments when the effect of lack of oxygen was most pronounced (there being a very short time interval between anaphase and cleavage). Of course the period when most oxygen is consumed need not necessarily be the period when the lack of oxygen is most felt, but it is of interest that the two seem to harmonize to some extent.

The peculiar cleavage figures following readmission of oxygen to cells deprived of it during the metaphase are somewhat similar to those obtained by Wilson ('01) after recovery from ether, though much more pronounced. Just what is the meaning of the irregular cleavage, it is difficult to say, except perhaps that it is a readjustment after a very profound disturbance to the dividing cell due to lack of oxygen. There is no doubt that very extensive changes can be made in the early cleavages without affecting later development, as has been previously shown in many ways.

Summary.

1. Observations were made on the living eggs of *Echinus microtuberculatus* and *Strongylocentrotus lividus* in a hanging drop

in a modified Engelmann's chamber. When the eggs are deprived of oxygen at any stage from fertilization to the completion of the first cleavage, development is arrested, but on readmission of air, continues and normal larvæ result.

2. When deprived of oxygen immediately after fertilization, the sperm aster gradually fades out and when air is readmitted, the aster reappears in the same position.

3. When deprived of oxygen at any later stage up to the metaphase, the astral fibers disappear but reappear on readmission of oxygen in the same position. Eggs have been as long as three hours without oxygen.

4. When deprived of oxygen at the full metaphase, the astral fibers disappear but sometimes not till after two hours. When air is admitted, the mitotic figure often appears as a white streak between two elongated astrospheres. Subsequent development is very irregular, often many asters appearing and cleavage planes dividing off large or small cells and subsequently often becoming obliterated, and other cleavage planes forming. After a while the eggs right themselves, giving rise to regular normal blastulæ and larvæ.

5. The egg may elongate and cleavage planes may start to come in at first during lack of oxygen, but the egg rounds out and the division plane is obliterated if kept longer without oxygen. These eggs proceed with irregular cleavages when air is admitted, but result in normal blastulæ.

6. If kept without oxygen at any stage after completion of the first cleavage to the metaphase of the second, normal cleavages result on admission of air.

7. If kept without oxygen just before second cleavage, when air is admitted, irregular divisions and fusions take place, but normal blastulæ result.

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PLATE I.

The drawings were all made with a camera lucida of living eggs under a Zeiss DD lens, ocular 2.

FIG. 1. Egg of *Echinus* immediately after fertilization.

FIG. 2. Same subjected to H for 20 min.

FIG. 3. Same after two hours in H.

FIG. 4. Usual appearance of egg after 40–60 min. in H, showing irregular outline and crinkling.

FIG. 5. Same after 1–3 hours in H.

FIG. 6. Same 50 min. after readmission of air.

FIG. 7. Egg of *Echinus* subjected to H at metaphase for 3½ hrs.

FIG. 8. Same egg 45 min. after air is readmitted.

FIG. 9. Same egg 25 min. later.

FIG. 10. Same 5 min. later.

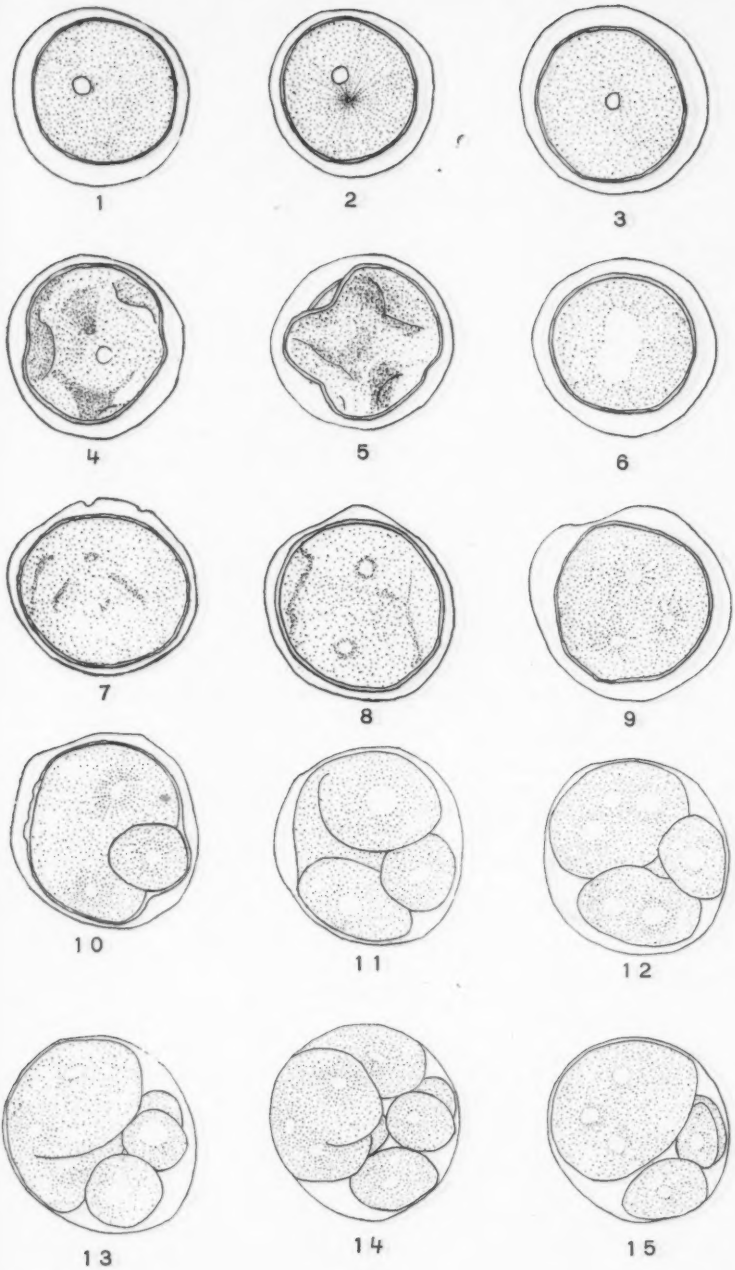
FIG. 11. Same 5 min. later.

FIG. 12. Same 30 min. later.

FIG. 13. Same 10 min. later.

FIG. 14. Same 5 min. later.

FIG. 15. Same 15 min. later showing fusion of cells.



ETHEL BROWNE HARVEY.

PLATE II.

FIG. 16. Same 20 min. later.

FIG. 17. Same 10 min. later.

FIG. 18. Same 15 min. later.

FIG. 19. Egg of *Echinus* subjected to H at metaphase for 1 hr. then air for 15 min.

FIG. 20. Same 20 min. later.

FIG. 21. Same 20 min. later.

FIG. 22. Same 20 min. later.

FIG. 23. Same 20 min. later.

FIG. 24. Egg of *Strongylocentrotus* subjected to H at metaphase for 15 min., then air admitted for 30 min.

FIG. 25. Same egg 20 min. later.

FIG. 26. Same 20 min. later.

FIG. 27. Same 2½ hrs. later.

FIG. 28. Same 15 min. later.

FIG. 29. Same 15 min. later.

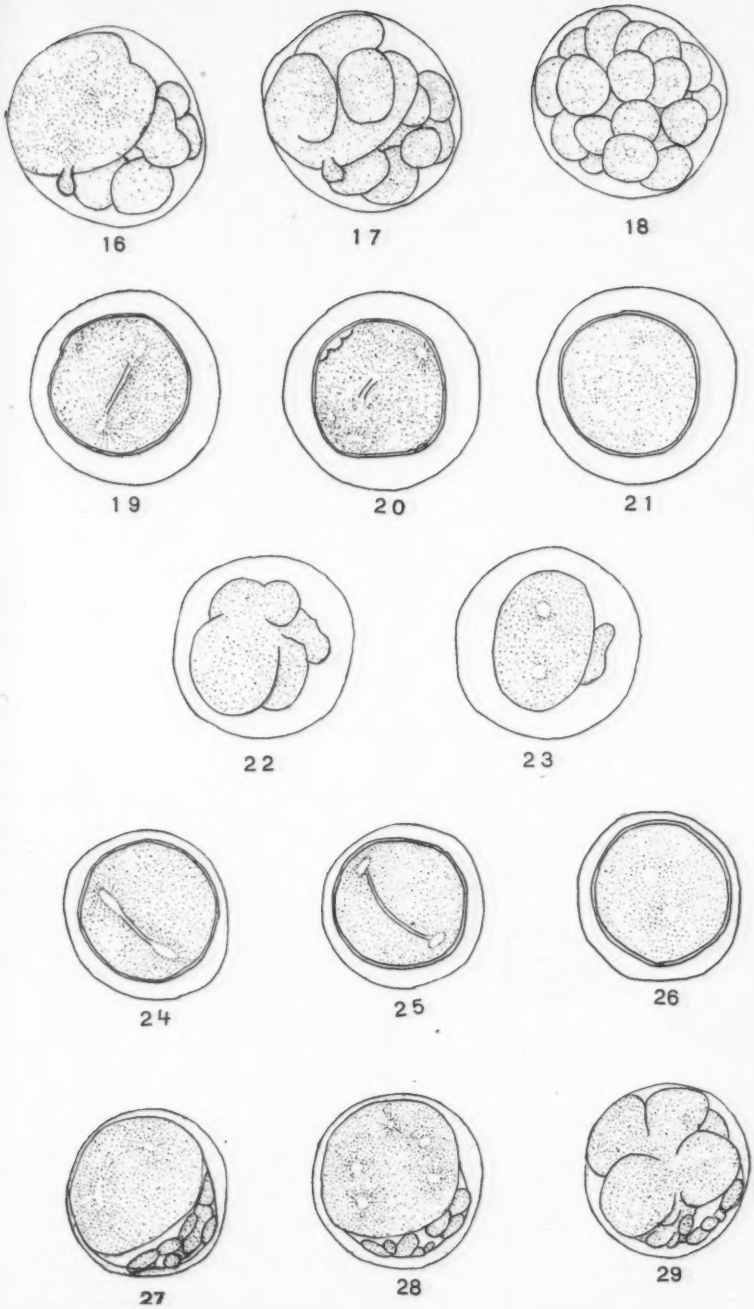


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PLATE III.

FIG. 30. Same 20 min. later.

FIG. 31. Same 1 hr. later.

FIG. 32. Another egg of *Strongylocentrotus* subjected to H at metaphase for 15 min., then air admitted for 3½ hrs.

FIG. 33. Same 10 min. later.

FIG. 34. Same 30 min. later.

FIG. 35. Same 20 min. later.

FIG. 36. *Echinus* egg subjected to H for 20 min. in anaphase.

FIG. 37. Same egg still under H 15 min. later.

FIG. 38. Same egg still under H 30 min. later.

FIG. 39. *Echinus* egg subjected to H for 17 min. in anaphase.

FIG. 40. Same egg still under H a few min. later.

FIG. 41. Same egg still under H 30 min. later.

FIG. 42. *Echinus* egg divided while subjected to H for 15 min.

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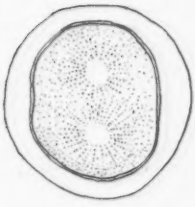

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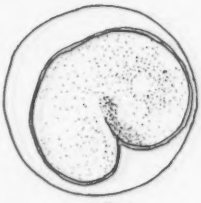

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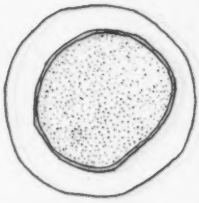

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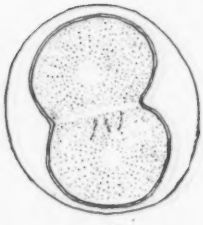

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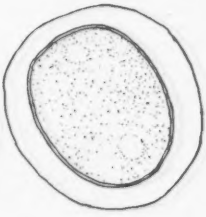

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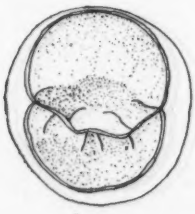

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UNIV. OF MICH.

THE COMPOSITION OF WOODS HOLE SEA WATER.

IRVINE H. PAGE.[1]

There are no more fundamental quantitative data than those regarding the composition of the medium nurturing the organisms on which much of the study of biological laboratories is based. Analyses of the tank water in the Marine Biological Laboratory were therefore made using the methods briefly outlined below.

Calcium and Magnesium.—50 cc. samples of sea water were boiled with a little HCl to expel CO_2, cooled and the calcium precipitated as oxalate, the oxalate subsequently ignited and weighed as CaO. Magnesium was precipitated in the filtrate with Na_2HPO_4 in the presence of an excess NH_4OH, ignited and weighed as the pyrophosphate.

Potassium was determined in 1 cc. samples of water by the method of Kramer and Tisdall (1). This method essentially consists in the precipitation of potassium with cobalti-nitrite reagent, and titration of the precipitate after special washing with $KMnO_4$. The method has been found to be exceedingly accurate and therefore has many advantages over the old, classical and tedious, precipitation as the chloroplatinate or the perchlorate.

Sodium was estimated by a modified Kramer and Gittleman technique (2). We have found that modification of the original technique—which was designed for blood serum only and for this most useful—is necessary because both calcium and magnesium are precipitated practically quantitatively by the sodium reagent, and are titrated along with the sodium.

The modified method consists in preliminary precipitation of the calcium and magnesium with alkaline phosphate, centrifuging off the precipitate, concentrating the supernatant liquor to about 3 cc. on the water bath, cooling and adding 10 cc. of the alkaline potassium pyroantimonate reagent, then 3 cc. of redistilled (over KOH) ethyl alcohol drop by drop with vigorous shaking. This precipitation is carried out in 50 cc. conical bottom Pyrex centrifuge tubes; it is important that these be used. After one half hour standing the tubes are centrifuged and the procedure carried out as Kramer and Gittleman recommend.

[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

Removal of the calcium as carbonate is unsatisfactory because of the very marked tendency of $CaCO_3$ to form supersaturated solutions. Gentle heating of the solution will, however, bring the carbonate down.

Oxalate precipitation seems quite satisfactory followed by phosphate for the magnesium precipitation, but here two steps are required and there is slight danger of carrying some oxalate along until the thiosulphate titration.

Using the above slight modification we have been able to get most accurate check analyses of known solutions of NaCl and $CaCl_2$. Kramer and Gittleman's method is a most useful and unique technique and seems to have a wide applicability for the direct determination of sodium.

As in the case of potassium we feel that the direct determination of sodium as compared with the determination by difference, as the combined sulphates, etc., has certain advantages.

Chlorine was estimated by the $AgNO_3$-$KCrO_4$ titration of Fr. Mohr.

Sulphate was precipitated as $BaSO_4$, ignited and weighed.

Phosphate was determined in 5 cc. samples of water by preliminary oxidation of organic matter with H_2SO_4 and H_2O_2 as described in more detail by Page (3), followed by the Benedict-Theis (4) technique. This technique consists essentially in the colorimetric estimation of a stable blue color developed by molybdic acid, hydroquinine and sodium bisulphite in the presence of a large excess of H_2SO_4. The following table gives the average of 4 analyses taken during the month of August 1926 from the tank water of the laboratory.

TABLE I.

ANALYSIS OF TANK WATER OF MARINE BIOLOGICAL LABORATORY.

Grams per 1000 cc. sea water at 20 degrees C.
Specific Gravity = 1.0180 (20 degrees C.).

		Millimoles.
Sodium	8.80	0.3826
Potassium	0.412	0.0105
Calcium	0.428	0.0107
Magnesium	1.3004	0.0534
Chlorine	18.350	0.5912
Sulphate	2.615	0.0272
Phosphate	0.002	—

Discussion.

These analyses are in rather close agreement with those of Wheeler (5) made at Beaufort, North Carolina. The sodium and specific gravity are slightly lower than most of Wheeler's and Ditmar's (6) analyses. Whether the lower sodium is due to an error in one or other of the methods we cannot say.

The phosphate showed the marked seasonal variations first carefully investigated by Atkins (7). At times we could find no determinable quantity present. Atkins has shown that these variations are largely dependent on the plankton growth especially the diatoms.

F. W. Clark (8) gives a summary of most determinations that have been made of the composition of the oceanic salts.

Summary.

1. The relative quantities of the more important salts in the Woods Hole, Massachusetts, sea water have been determined.

2. The Kramer-Gittleman technique.for the determination of sodium in serum has been modified for the analysis of sea water. It has been shown that Mg and Ca interfere seriously when present in quantity and must be removed before the Na determination.

It is a pleasure to acknowledge my indebtedness to Dr. W. A. Perlzweig for his many valuable suggestions.

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THE OILS OF THE SEA URCHIN AND STAR FISH EGG.

IRVINE H. PAGE.[1]

The fatty fraction of the Echinoderm ova is particularly important because there is evidence that this fraction furnishes much of the energy requirement for the development of the egg. The sterol content of the eggs has been determined and a new sterol found in the star fish egg (*Asterias forbesii*) by Page (1). The sea urchin (*Arbacia punctulata*) egg was shown to contain a rather large quantity of true cholesterol. It seemed desirable now to examine the oil contained in these eggs, the results of which analysis are presented in this paper.

Oil Content.

The eggs were removed from the ovaries, strained, and strongly centrifuged to remove as much sea water as was possible. They were then placed in a flask and extracted first with an alcohol-ether mixture followed by repeated ether extractions for eight days at 40 degrees C. in a thermostat. Some of the excess ether was allowed to evaporate at room temperature and then acetone added in large excess in order to precipitate the acetone insoluble phosphatide fractions. After standing over night the mixture was filtered and the solvent from the filtrate allowed to evaporate at room temperature. The residue from this evaporation was placed in a $CaCl_2$ desiccator and the desiccator kept in the thermostat at 40 degrees C. for 4 days. The residual oily material was then subjected to the identification procedure detailed below. The phosphatide fraction was dried at 100 degrees C. and weighed.

Protocols.

Arbacia Oil.—The oil had a brownish red color and smelled strongly of lower fatty acids. The material had a heavy consistency and tended to lump together. It was freely miscible

[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

with CCl_4 chloroform, ether, and acetone. From its appearance on standing it seemed to behave somewhat as a semi-drying oil. 8.3 grams of oil was obtained from 183 million eggs. The iodine number as determined by the Wijs method was 146–148, and the saponcifiation value (Kottstörfer value) approximately 606.0.

The I_2 value is suggestive of the marine animal oils such as Menhaden and Sardine oils, also the liver oils. The high saponification value confirms the results of the volatile acid determination presented in Table I. It also suggests that much of the oil represents fatty acids of low molecular weight.

The fatty acids volatile with steam were determined by the method of Dyer (2). Twelve million *Arbacia* eggs were used. The results of the steam distillation of star fish eggs is also included. 9,160,000 of these eggs were distilled. Three hours and forty minutes were required in both cases for the distillation.

TABLE I.

DYER METHOD FOR STEAM VOLATILE FATTY ACIDS OF *Arbacia* AND *Asterias*.

Fraction.	Amount of Distillate *Arbacia*.	Amount of Distillate *Asterias*.	Cc. *N*/10 NaOH Required to Neutralize *Arbacia*.	Cc. *N*/10 NaOH Required to Neutralize *Asterias*.
1.....	13.3	7	.346	.175
2.....	24.0	14.0	.72	.350
3.....	22.0	23.9	.44	.597
4.....	20.5	19.2	.246	.480
5.....	32.0	20.7	.384	.414
6.....	18.0	34.0	.18	.510
7.....	20.0	33.5	.20	.335
8.....	26.0	39.2	.26	.196
9.....	17.0	36.6	.17	.219
10.....	56.0	43.0	.56	.215
11.....	47.0	55.0	.25	.220
12.....	40.0	13.5	.24	.067
Total.....	335.8	339.6	4.001	3.778

6,000,000 *Asterias* eggs = 2.474 cc. *N*/10 NaOH.
6,000,000 *Arbacia* eggs = 2.000 cc. *N*/10 NaOH.
Since the *Arbacia* eggs = 75 μ in diameter and *Asterias* eggs = 104 μ in diameter.

Calculating *Arbacia* volume to that of *Asterias* = 2.527 cc. *N*/10 NaOH for 6,000,000 *Arbacia* eggs.

Asterias Oil.—An orange colored oil, non-drying, pungent odor very much like vanillin, which has about the consistency of olive oil. The I_2 number (Wijs) was approximately 110–115 and saponification number 318.8. The saponification number and the physical properties of the oil suggest that a much greater proportion of high molecular weight fatty acids are present in this oil than in that of *Arbacia*.

The *Asterias* oil is present in great abundance while *Arbacia* contains much less oil. This fact seems important. Distillation of *Asterias* eggs with KOH gives very appreciable amounts of H_2S. Very little resulted from the distillation of *Arbacia*.

Phosphatide Fractions.

1.539 grams of acetone insoluble material was obtained from 183 million *Arbacia* eggs. Much of this precipitate was insoluble in boiling alcohol suggesting a high percentage of kephalin. The presence of sphingomyelin was indicated by the presence of a small amount of white precipitate on the addition of ether to the alcohol solution.

Acetone precipitates a large quantity of brownish white gummy material from *Asterias* egg extract. It was largely soluble in hot alcohol suggestive of a large proportion of lecithin. Further examination of this fraction has been deferred.

It is interesting to note that qualitatively large amounts of soaps were found especially in the *Asterias* egg.

Summary.

1. The oil of the sea urchin egg (*Arbacia punctulata*) has an I_2 number of 146–148 and saponification value of approximately 606. The star fish (*Asterias forbesii*) egg oil I_2 number is about 110–115 and saponification value 318.8.

2. Steam distillation of the eggs by the Dyer method suggests along with the Köttstorfer number that the fatty acids of the *Arbacia* egg are of a lower order than those of the *Asterias* egg.

3. Qualitatively there appears to be more kephalin in the *Arbacia* egg and more lecithin in the *Asterias*. The *Asterias* egg

appears to contain more KOH decomposable sulphur compounds than the *Arbacia* egg.

4. Qualitatively large quantities of soaps were found.

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THE ELECTROLYTE CONTENT OF THE SEA URCHIN AND STAR FISH EGG.

IRVINE H. PAGE.[1]

The sea urchin (*Arbacia punctulata*) and star fish (*Asterias forbesii*) eggs have been the subject of very extensive morphological and physiological research. Since both of these types of investigation are intimately related to the chemical composition of the egg, the following analyses of the electrolyte content of these two Echinoderm ova are presented.

Experimental Methods.

Arbacia eggs were removed from the ovaries, strained through cheese cloth, centrifuged until as much sea water as possible was removed. The egg mass was then almost solid. The surface layer was then quickly washed over with a few cubic centimeters of distilled water, not sufficient to cause cytolysis but enough to remove any slight amount of sea water remaining on the surface. A sample of the egg mass was removed to determine the number of eggs, the volume of the egg mass determined, and the mass dried at 100 degrees C. in a Freas oven until constant in weight. Samples of this dried material were then ashed in a platinum crucible in an electric crucible furnace, a few drops of HNO_3 being added to aid the ignition. The ash was weighed and dissolved in 5 cc. of $N/10$ HCl, made up to 25 cc., and the SiO_2 centrifuged out. Aliquot portions were then used for the special analytical procedures.

Calcium.—5 cc. of the filtrate were brought to about pH 6.0, 2 cc. saturated ammonium oxalate added and the centrifuge tube in which the solution was contained vigorously "rubbed down" with a stirring rod. After 12 hours the tube was centrifuged and the precipitate washed three times with hot water. The calcium was then determined by the usual ignition method as CaO and as an alternative procedure dissolved in 4N H_2SO_4 and titrated hot with $N/100$ $KMnO_4$.

[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole. Mass.

Magnesium.—The filtrate from the above proceedure was then made strongly alkaline with concentrated NH_4OH, and an excess of a solution of K_2HPO_4 added, the solution allowed to stand overnight, the precipitate filtered off on ashless filter paper, washed with dilute NH_4OH, ignited strongly, and weighed as $Mg_2P_2O_7$.

Sodium.—The filtrate from the above procedures was then concentrated on a water bath to about 3 cc., made alkaline to phenolphthalein with sodium free 10 per cent. KOH, 10 cc. of potassium pyroantimonate reagent added and the procedure further carried out as recommended by Kramer and Gittlemann (1). Sodium cannot be determined directly in the presence of fairly large amounts of Ca or Mg because these two elements are precipitated by the pyroantimonate reagent and may be titrated as sodium.

Potassium.—2 cc. samples from the original filtrate were analysed for potassium by the method of Kramer and Tisdall (2).

Iron was estimated colorimetrically by the method of W. S. Allen, using salicylic acid to develop the color as described by Scott (3).

Sulphate determined as $BaSO_4$ following hot $BaCl_2$ precipitation.

Chlorides were titrated using the Fr. Mohr method.

Total Phosphorus and Lipoid Phosphorus was determined by the Benedict-Theis method (4) modified for egg analysis as described by Page (5).

Nitrates were estimated by the phenolsulphonic acid method ordinarily used in water analysis.

The above analyses were then carried out on filtrates prepared as follows: Samples of one million eggs were centrifuged to remove as much sea water as possible, made up to a 3 cc. mark on the centrifuge tube with distilled water, 3 cc. more distilled water added and the tube vigorously shaken to cytolyse the eggs. After standing 15 minutes 4 cc. of 20 per cent. trichloroacetic acid was added and approximately 0.2 grams of prepared bone black,[1] again shaken and allowed to stand 15 minutes. The

[1] Commercial bone black boiled with HCl, thrown onto a Büchner filter and washed until the filtrate was chloride free.

mixture was then filtered through ashless filter paper and the filtrate used for analysis. The bone black should have removed the majority of the echinochrome.

Results.

Table I presents the average figure for the substance sought. In every case at least three analyses were done. The results are calculated on the basis of milligrams per one million eggs.

Table I.

Electrolyte Content of One Million *Arbacia* Eggs.

Radicale.	Mg. per Million Eggs.	Millimols.	Milliequivalents.
Calcium	1.90	0.047	0.094
Magnesium	4.48	0.182	0.364
Sodium	1.301	0.056	0.056
Potassium	2.445	0.063	0.063
Iron	0.030	0.0005	0.0015
		0.348 Total cation	0.5785 Total cation
Sulphate	0.00046	0.00004	0.00008
Chloride	0.1864	0.0053	0.0053
Total phosphate	0.9064	0.0291	0.0873
Nitrate	Trace		
		0.0344 Total anion	0.0927 Total anion

The weight per million eggs of the material dried to constant weight at 100 degrees C. was approximately 0.124 grams, and the ash was 8.5–10 per cent. of the dry weight of eggs. The *Arbacia* egg averages 74.1 μ in diameter (Glaser (6)).

SiO_2 was not quantitatively determined but qualitatively rather large amounts were found present.

The distribution of the phosphorus between lipoid and inorganic is discussed in another paper (page (7)).

The analysis of *Asterias* eggs was confined to the analysis of the trichloroacetic acid filtrates described above. Due to the greater size of the *Asterias* egg (103.6 micro for unfertilized eggs (Glaser (6))) only 250,000 egg samples were analyzed. The volume relation (4.1893) of the *Arbacia* and *Asterias* egg is 2.1–5.2 or one million—404,000 eggs, hence although for convenience

250,000 eggs were analyzed, the results are calculated to a basis of 404,000 eggs for comparative purposes. At least two analyses were done for each element determined but the results are statistically not so accurate as the *Arbacia* analyses due to the relatively small number of estimations. The relative values are probably more accurate than the absolute.

TABLE II.

ELECTROLYTE CONTENT OF THE *Asterias* EGG.

Radical.	Mgs. per 404.000 Eggs.
Calcium	1.487
Magnesium	2.101
Sodium	4.84–7.27
Potassium	6.77
Lipoid phosphorus	0.541
Acid soluble phosphorus	0.591

Due to the incompleteness of the data in Table II the milli-equivalents are not calculated.

DISCUSSION.

In both the star fish and sea urchin egg the potassium is higher than the sodium although in the sea water of Woods Hole the sodium is much higher than the potassium. (See page (8) for analysis of Woods Hole sea water.) The Ca : Mg in the sea urchin egg is approximately 1 : 2 and in the star fish egg the Mg in the ratio is slightly lower than 2. Total phosphorus is slightly higher in the star fish than sea urchin but the distribution is about the same between lipoid and acid soluble. (See also page (7).) Sulphate in the sea urchin is very low.

Using the salicylic acid method for iron determination the values found for *Arbacia punctulata* are higher than those found by Warburg (9) for *Strongylocentrotus lividus*. We find 0.24 mgs. per gram of dried eggs whereas his value is only 0.01 mg. per gram.

The fact that the cation equivalent is much higher than the anion opens an interesting field for speculation. Presumably much of this excess cation is combined in the lipoid phase and some held by proteins. Certainly much of it must be osmotically inactive.

My thanks are due Dr. G. H. A. Clowes and Dr. W. H. Perlzweig for suggestions during the course of this investigation.

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THE REACTION OF HYDRA TO INANITION.

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During the past fifteen years the senior author has observed that Hydra, kept in laboratory aquaria to which food has not been added, decreased greatly in size. In this general or casual observation, it was further noticed that green Hydras survived under these adverse conditions for much longer periods than did Hydras of other species. This has been a general experience in this laboratory, though it stands in sharp contrast with Wagner's ('04) statement that "Green Hydras stand starving very poorly, usually perishing in two or three days" (p. 612). In later years it was observed further that the poorly fed Hydras would lose parts of their tentacles. Sometimes the full complement of tentacles has disappeared. During the last three years, we have been seeking to learn the conditions under which Hydras lose their tentacles.

The literature presents the following causes by which Hydras lose their tentacles. N. Annondale ('07), in studying *Hydra orientalis*, found a seasonal variation from four to six tentacles. During the hot season this Hydra has but four tentacles, while during the cold part of the year it has six tentacles. G. Entz ('12) observed that an infection with *Amœba hydroxena* may lead to a complete loss of tentacles. E. Reukauf ('12) observed that the ciliate *Prodon teres* caused Hydra to lose its tentacles. Again, the presence of another ciliate, *Kerona pediculus*, according to P. Schultze ('13), leads to hypertrophy of the tentacles. E. Schultz ('06) observed that hunger set up a process of dedifferentiation within the tentacles of Hydra; just as Huxley and De Beer ('23) found that hunger and toxins caused dedifferentiation and resorption of the tentacles of *Obelia* and *Campanulana*. Huxley and De Beer observed that this process of dedifferentiation and resorption might involve, not only the tentacles, but also part of the zoöid. Finally Berninger ('10) observed that, in response to inanition, Hydra lost its tentacles; but he did not see the manner in which they were lost.

We have found that Hydra is very susceptible to the impulses arising from scarcity of food. Hydras, at the time they are taken from the pond, present sometimes one or more fragmentary tentacles. That these do not represent mere accidental losses is suggested by the fact that in a flourishing population of Hydras one does not readily meet with such imperfect specimens, whereas when the population of a pond is on the decline specimens with incomplete tentacles are quite frequently encountered.

That Hydras react rather readily to hunger is further indicated by the observations of Wilson ('91). He records that "After the exhaustion of the food-animals—which takes place rapidly when the Hydras become very numerous—the Hydras are compelled to live wholly upon mud" or sediment at the bottom. In this sediment they find fragments of plant tissue and numerous minute Infusoria. The polyps, "after descending, usually gorge themselves with the sediment at the bottom." Welch and Loomis ('24) verified this observation. They found, however, "that the Hydras did not always thrive indefinitely on the sediment material dredged from the deepest parts of the lake," p. 227.

Realizing, then, the susceptibility of Hydra to hunger, we set to work to see if inanition was a factor leading to the loss of tentacles. This effort was made through the observation of living isolated individuals and the histological study of isolated individuals and controls.

Hydra has been observed taking into its body a tentacle's end as it clung to the food. This has been seen frequently. For example Mr. J. W. Nuttycombe saw a Hydra swallow the ends of two tentacles that had been involved in the capture of a Cyclops. After considerable struggle on the part of both the tentacles and the body-proper, these tentacles were thrown from the cœlenteron. So far as could be determined these tentacles had not been injured. In such examples, an effort to free the tentacle from the cœlenteric cavity is always apparent.

In contrast to this, Mr. M. C. Yoder and the senior author observed an isolated Hydra, that had been deprived of food, hold a tentacle within the cœlenteric cavity for twenty minutes. Throughout this period the tentacle remained quiet. There was

in this case no effort to free the ingested tentacle. After twenty minutes, however, the tentacle was seen to be slipping out of the mouth. When freed the tentacle presented a constricted zone about which the peristome had held it during the period of ingestion.

The history of this tentacle has an interesting bearing upon our observations; for it shows that the tentacle was not one that had undergone necrosis prior to its having been ingested, since it showed no eroded tissue and eventually fully recovered, leaving no trace of the constricted region. This tentacle, therefore, had not suffered necrosis before being ingested. Marshall ('23) makes this observation: "Some brown Hydras were induced to swallow pieces of *Hydra viridis* by slipping the latter inside the carapace of *Daphnia*, but they were ejected along with the remains of food," p. 614. The suggestion may be taken that only dead Hydra-tissue might be freely ingested since Marshall, in this case, saw fresh Hydra tissue "ejected along with the remains of food."

But our observations do not lend support to this negative inference. Miss Edna McNally in 1923 observed an *Hydra viridis* "put the end of one of its tentacles into its mouth." The end of the tentacle could be clearly seen inside of the cœlenteron. After the tentacle had been kept in the cœlenteron about fifteen or twenty minutes, the Hydra dragged the shortened tentacle with a ragged end from the mouth, leaving part of it within the cœlenteron.

In addition to having actually seen an Hydra ingest a piece of its tentacle, we have observed a green specimen, which during sustained inanition had but recently lost parts of some of its tentacles, throw out from its mouth defragmented tissue of green Hydra. On one occasion, after such cellular material had been regurgitated, it was again ingested.

Thus, actual observation of living individuals has yielded evidence that Hydras, that have been deprived of food, feed upon their tentacles.

Further evidence was secured through isolating two series of individuals. By depriving one of these series of food and feeding the other series well, we have secured some interesting results.

On March 14, 1925, six individuals were isolated in six watch

glasses and given no food. Another lot of six were isolated. These were used as controls and were kept supplied with food in the form of *Cyclops* and daphnids. The observations were kept up until April 13, during which time, one of the controls had died (March 22) but none of these controls had lost part of their tentacles. Except for the one specimen dying, the controls at the end of the period of observation were in excellent condition and showed no negative features. The starving specimens presented a marked contrast to the control series for by March 22 two specimens had died. Of these two that had died, one had lost more than two thirds the length of each of its tentacles the day before it had died. The four other specimens, by April 14, had lost parts of their tentacles. The most conspicuous individual in the starved series was designated specimen six in our records. Figure 5, *A*, represents the contour of this animal when expanded at the time of isolation. On March 24 it showed, in the expanded condition, that its tentacles were shorter and knobbed on the end (Fig. 5, *B*). After that not much change could be noted, except that the knobbed condition of the tentacles had disappeared, until March 30. Then it was observed that a great amount of the tentacles had been lost (Fig. 5, *C*). On March 31, but mere stumps of the tentacles remained (Fig. 5, *D*). The specimen on April 1, showed the bud of one tentacle making its appearance. By April 8, tentacles relatively as long as those shown at *A* in Fig. 5 were present. During the period of inanition the body, as a whole, had been reduced in size so that by April 10 the regenerated tentacles were relatively longer than those which were involved at the beginning of the observation (Fig. 5, *E*). On April 9, some parts of this second crop of tentacles had been eaten. On April 14, the specimen died.

In the above observations, no effort to control the factor, presented by the accumulation of toxic metabolic products, was made. It is quite possible that such effort need not have been made, for the fed Hydras had grown in size and, therefore, must have thrown a greater amount of toxic material into the water than did the unfed specimens. In addition to this, the presence of the *Cyclops* and daphnids added much to the concentration of metabolic toxins in the water of the fed specimens as compared

to the water in which the unfed specimens were kept. However, a second series of observations was made. In this series, five specimens were isolated and carried through unfed while five isolated specimens were fed to serve as controls. Each day the water in all watch-glasses containing Hydras was areated by blowing air through it and every twenty-four hours or every forty-eight hours the old water was drawn off and fresh spring water added. In these instances, we got just as conspicuous contrast between the unfed series and the fed series as has been described for the series running from March 14 to April 13. The most pronounced example, obtained in this manner of Hydra's reaction to inanition, was obtained from specimen 4 of a series begun October 6, 1925, and ending October 30. A camera lucida drawing was made of this specimen as it hung from the surface of the water in the watch-glass, October 6 (Fig. 6). Three days later the camera lucida drawing shows that there had been a marked reduction in the size of the specimen (Fig. 6). On October 11, the specimen presented only mere stumps of tentacles (Fig. 6). Camera lucida outlines made daily showed that those tentacle stumps gradually grew until by October 22 the camera lucida outline reveals that the now smaller Hydra had regenerated seven tapering tentacles (Fig. 6). The next day, however, one of these tentacles had disappeared (Fig. 6). A second tentacle was missing October 24 (Fig. 6). Two days later the camera lucida drawing indicates that the specimen had lost the greater part of each of its tentacles (Fig. 6). In this condition the specimen remained as a listless individual for three days and then died.

The above specimens were examined daily for the presence of parasites such as *Amœba* and other protozoa and were thus determined to have been free of such infection. The abruptness, however, with which a part of a tentacle or a whole complement of tentacles would be lost did not suggest that these tentacles disappeared through attack by parasites or by degeneration and resorption. It suggests rather that the specimens had been feeding upon their tentacles.

The histological evidence supports the idea that dedifferentiation, degeneration and parasites were not involved.

Specimen "369—2—a" had been isolated in a hanging drop until it had lost its tentacles. The slide made from this specimen bears a complete series of sections in which the shortened tentacles show no erosion such as *Amœba* and other parasites cause. Moreover, all of our slides show no dedifferentiation processes. We have slides that show wound scars at the end of tentacular stumps but in which the ectoderm is laid down as a continuous sheet about the tip (Example "370—2").

But our histological evidence leads further to the conviction that tentacle-material is ingested and digested. Mr. Looper, of this laboratory, has two individuals sectioned, in one of which the sections show a series of sections of three fragments of tentacles lying within the cœlenteron, while the other shows but one fragment of tentacle lying within the cœlenteron (Looper's slides "2 ovum la," and "Hydra Ovum 11").

The transverse sections of the tentacular fragments, that are to be found in sections of polyps that have suffered inanition, show a diploblastic pattern but present very poor detail. In Fig. 4 we have a transverse section of an ingested tentacular fragment. The region of the endodermal epithelio-muscular cells is most greatly broken down (Fig. 4, *b*). What we take to be residia of secreting endodermal cells are indicated at *a*. The ectoderm seems to be least attacked by the digestive fluids of the cœlenteron. All elements here, too, have been greatly shrunken.

Not only do the histological specimens show the tentacular fragments lying within the cœlenteron but they indicate that the material, thus taken in, is digested and absorbed.

This was further checked by fixing specimens by the pond in which a vigorous population of Hydras was growing and contrasting the histology of these with that of the isolated specimens that had been kept under daily observation in the laboratory. Slides, bearing material taken from the vigorous population of a pond, show no Hydra-tissue within the cœlenteron and very few, if any, nematocysts within the epithelio-muscular cells of the endoderm. In contrast to this, the histology of specimen "375" is significant. This specimen was isolated in a Petri dish, containing spring water, at 3:30 P.M., October 28, 1921. At 9:25 A.M. the next day it was found to have had but the merest

stumps of tentacles. Our slide shows the tentacular stumps involving but a few sections of the series, thus recording their small size. Hydra-tissue, being digested within the cœlenteron, is found in these sections. While within the endoderm's epithelio-muscular cells are *many* nematocysts.

Another specimen, "369—1," was kept isolated within a hanging drop for three days and examined daily. At the end of this period, it was found to be in a listless condition and to have very short tentacles. This Hydra, in the fixed and sectioned condition, revealed many nematocysts within the cœlenteron and food-vacuoles of the epithelio-muscular cells of the endoderm.

Specimen "380" was isolated in a test tube. This tube was kept partly submerged in the pool from which the Hydra had been taken. Daily inspection revealed that on the seventh day the tentacles were blunt, their ends ragged, no amœbas or other parasites present. The series of sections on our slide 380 shows almost wholly digested hydra-tissue within the cœlenteron, there being left, as yet, naked nuclei, at one side of the dissolving mass, and a cnidoblast, in bad condition, surrounding a "stinging" nematocyst. In addition to this there are many nematocysts in various phases of digestion within endodermal cells.

These observations indicate that, not only are the protoplasmic constituents of the ingested fragments of tentacles digested and absorbed, but that even the nematocysts are likewise appropriated as food.

The fact that these structures can be digested by Hydra is interesting. Glaser and Sparrow ('09) found that "in the case of peptic digestions, all the tissues except the nematocysts, were dissolved," p. 362. But it has since been observed that parasitic ciliates and amœba can digest the nematocysts of Hydra. The histology of Microstoma's enteron or "intestine" greatly resembles that of Hydra and it does not digest the nematocysts but hands them over for offensive or defensive purposes to the mesenchyme. Hydra's immediate demand, during inanition, is neither defensive nor offensive but nutritional, so it digests the nematocysts. Fig. 1 shows a nematocyst from which the cnidoblast has already disappeared. Except for the lacking cnidoblast the nematocyst, as it lies within the food vacuole of

an endodermal epithelio-muscular cell, does not present a markedly unusual appearance. In Fig. 2, however, the enclosed thread of the nematocyst displays marked shortening and the nematocyst, as a whole, takes the stain less readily. In Fig. 3, we have a contrast presented between a nematocyst that has been almost digested and one lying in the usual position in the ectoderm. It is seen here that the nematocyst within the endodermal cell has been almost wholly dissolved. Eventually no trace of the ingested nematocyst is to be found. The hungry Hydra, therefore, consumes not only the readily digestible tissues of its tentacles but also the nematocysts. From the material, thus obtained, energy is derived upon which the life of the polyp is tided through a period of inanition and by which the polyp is enabled to develop a new complement of tentacles.

An interesting feature of this process is presented in the fact that the stumps of tentacles receive relatively much more of the ingested tentacular material than do other parts of the body Our slide "375" shows this clearly. This slide bears in series the sections of an Hydra that had been isolated in a Petri dish in the laboratory for eighteen hours. At the end of this period but small stumps of its tentacles had remained. In this series of transverse sections there are ninety-five sections.

The tentacles involve about twenty of these (10 microns) transverse sections. The endodermal cells in the sections that show the bases of the tentacle-stumps are heavily charged with food-vacuoles that contain nematocysts and hydra-cells. The endodermal cells of the tentacle-stumps have in this instance much more food supplied them than do the endodermal cells of the body proper. This is in contrast to what ordinarily takes place. If a complete Hydra be fed and later sectioned, the endodermal cells of the tentacles will be found to have relatively fewer food-vacuoles than the endodermal cells of the body-proper. So here again we have it suggested that in a diploblastic animal local needs must be locally met. In Hydra, having but two tissues there can be no circulatory medium. Therefore, when material is needed for the regeneration of tentacles it cannot be taken up by the general endoderm of the body-proper and then as lymph or plasma sent to the tentacular bases, but the

material must be handed directly, in the form of food, to the endoderm of the tentacle-bases.

SUMMARY.

Hydra, as a diploblastic animal, can have no circulatory medium. Perhaps for a similar reason it can have no storage tissue such as fat. Green Hydras seem able to fall back upon the surplus foods of its zoöchlorellæ, for, contrary to some observers, it has been our experience that *Hydra viridis* is much less influenced by inanition than other species.

In all species, if the inanition be prolonged and the individuals be kept free from parasites and concentrated toxins, each specimen not getting food, will begin to feed upon its tentacles. First the ends of the tentacles are bitten off. If now food is yet with-held from the Hydra, it will feed upon its tentacles until but the merest stumps are left.

The ingested tentacles will be digested. Even the nematocysts (both types) will be taken into food-vacuoles within the epithelio-muscular cells and be completely digested and absorbed.

After the Hydra has thus fed upon and appropriated its tentacles—except for mere stumps that stand about the peristome—the bases of the amputated tentacles will regenerate and the Hydra, now reduced in size, will possess a new group of tentacles.

In feeding upon the tentacles that have been ingested, the endoderm of the stumps of tentacles appropriate relatively more of the ingested tentacle-material than is appropriated by the endoderm of the body proper. This appears to be correlated with the fact that regeneration is to take place in the tentacular stumps.

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EXPLANATION OF PLATE.

FIG. 1. Interlocking secreting (*a*) and epithelio-muscular (*b*) cells of the endoderm. *b* bears a food vacuole (*c*) within which is a nematocyst that shows little distortion. × 1500.

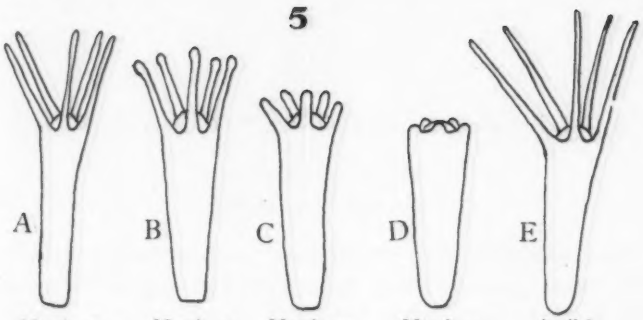
FIG. 2. An epithelio-muscular cell of endoderm bearing a food vacuole (*a*) within which is a nematocyst that shows advanced internal disintegration. × 1500.

FIG. 3. An epithelio-muscular cell of endoderm with adjacent ectodermal cells. A greatly broken down nematocyst within a food vacuole (*a*) is contrasted with a nematocyst in the usual position within the ectoderm (*b*). × 1500.

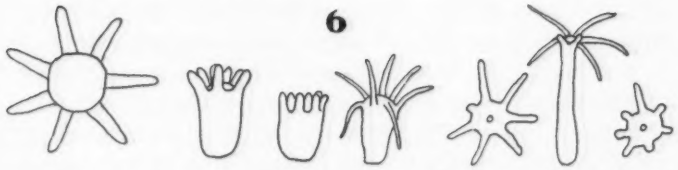
FIG. 4. One of the transverse sections of a tentacular fragment that lay in the cœlenteron of a sectioned Hydra from which food had been withheld. (*a*) residia of secreting endodermal cells; (*b*) residia of epithelio-muscular endodermal cells. × 750. From H. L. Firebaugh's slide "No. 15. 34.7–85.8."

FIG. 5. Series of free-hand outlines to indicate changes that had taken place in an individual *Hydra* from which food had been withheld.

FIG. 6. Series of camera-lucida outlines to indicate changes that had taken place in an individual *Hydra* from which food had been withheld.

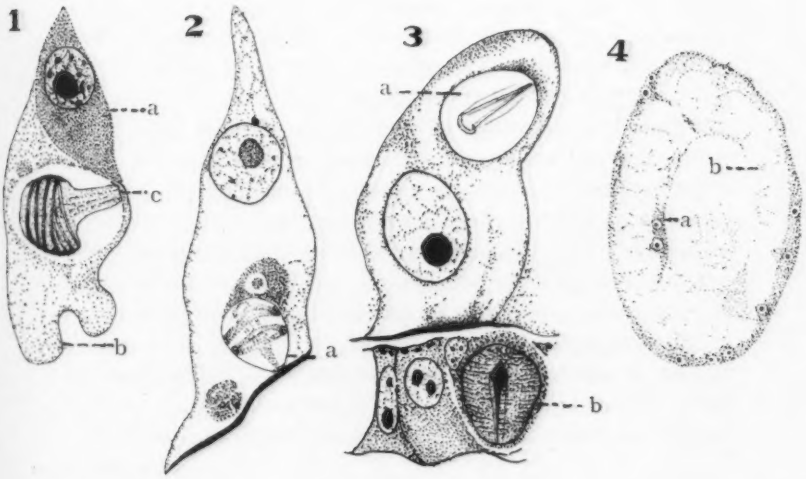


March 14 to March 23. | March 24 to March 29. | March 30. | March 31. | April 8.



Oct. 6. Contracted Aboral Aspect. | Oct. 9. Contracted. | Oct. 11. Contracted. | Oct. 22. Expanding. | Oct. 23. Contracting. | Oct. 24. Expanded. | Oct. 26. Contracted.

Oct. 30. Dead.



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CILIATED PITS OF *PRORHYNCHUS STAGNALIS.*

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The specimens used in this study were collected in the early months of the year in a very small, slowly running stream, the outlet of a spring, which is located about a mile west of the University of Virginia. They occurred in greatest abundance from twenty-five to forty feet below the spring, in the soft, oozy, decaying organic matter at the bottom of the stream. *Prorhynchus applanatus* was always found in equal, if not greater numbers, along with *Prorhynchus stagnalis*.

Some of the grass, weeds, and algae growing in the stream were also taken along with the sediment, and the whole collection placed in a large aquarium dish and left to stand for from twelve to twenty-four hours. The rhabdocœles appeared at the edge of the aquarium, near the water's surface, sometimes just beneath the surface film. When a film of bacteria developed at the surface of the water, the animals soon died, seldom being obtained five days after the collection had been in the laboratory. By keeping some of them in small dishes in spring water with some small amount of the decaying matter collected in the stream, and by adding fresh water every three or four days, specimens were kept alive for a maximum period of two weeks.

The body of *Prorhynchus stagnalis*, when moving through the water, is very slender or thread-like, being about the same width all along the body-length. When at rest, the posterior half is slightly broader, the average length of the body being about 6 mm.

The anterior region of *Prorhynchus stagnalis* is the exploratory region of the body. For, in response to a disturbance, the region of the body, anterior to the ciliated pits, of an animal that is quiet or of one that is creeping, is laterally expanded (Fig. I, *l.e.*) and swayed to and fro; while in addition to this the cilia of the pits, during disturbance, are set into active motion. The cilia of the ciliated pits are quiet when the animal is not excited or disturbed.

For histological study, specimens were fixed with greatest success in Bouin's fluid, cut from five microns to seven microns thick, and stained in iron hæmatoxylin, with Bordeau red or eosin as a counter stain. It was found best to handle the specimens in very small numbers at a time. While fixing, it was also found advantageous to keep the animal under the slight pressure of a cover glass to prevent the curling of the body. The best stain for differentiating nervous tissues was Mallory's connective-tissue stain, fixation being in Zenker's fluid. The nervous tissue is stained blue in this case.

Acknowledgment must be made to Dr. W. H. Taliaferro for a box of slides of *Prorhynchus stagnalis* which he left with Dr. Kepner, and which were of great aid in this work.

It is necessary to consider the central nervous system with which the two ciliated pits are associated before discussing the latter. There are two dorsal ganglia just anterior to the opening of the muscular pharynx as it lies at rest within the pharyngeal sheath (Figs. II and III, *d.g.*). These ganglia are connected across the pharyngeal sac by a relatively broad commissure (Figs. II and III, *d.c.*). Posteriorly from the dorsal ganglia extend latero-ventrally two nerves, which cannot be traced beyond the beginning of the enteron (Figs. II and III, *p.n.*). Anteriorly from the dorsal ganglia extend latero-ventrally two other nerves, which lie in contact with the mesial surfaces of the ciliated pits, and passing beyond the ciliated pits branch in the extreme anterior portion of the body (Figs. II and III, *a.n.*). There is another set of nerves connected with the pharynx, the exact path and relation of these nerves to the ganglia and pharynx having not been definitely determined.

From Kepner and Taliaferro's ('16) paper on "Organs of Special Sense of *Prorhynchus applanatus Kennel*," we find this comparison of the ciliated pits in *Microstoma caudatum* and *Prorhynchus applanatus:*

"As stated before, we consider the ciliated pits the chief organs of special sense. These organs open on the ventral side and are disposed laterally and ventrally to the thick nerve commissure which joins the two dorsal ganglia. . . . When the animal is crawling upon a surface, it seems to make numerous exploratory

movements by raising and lowering the anterior sixth of its body. Thus we have a crawling animal with ventrally disposed ciliated pits which makes its exploratory movements by raising and lowering the anterior end of its body. It is well to compare the conduct of this animal with that of *Microstoma caudatum Leidy* with reference to the position of their respective ciliated pits. *Microstoma caudatum* is a free-swimming animal and has laterally disposed pits. As we have shown ('12) this animal makes exploratory movements by moving its anterior end from side to side. Likewise, we gave experimental evidence to show that these exploratory movements were made in order to test the surrounding medium. Thus we see by the comparison of the two rhabdocœles that the method in which they test the surrounding water conforms to the position of their ciliated pits."

So much for *Microstoma caudatum* and *Prorhynchus applanatus*. Kepner and Taliaferro in *Microstoma caudatum* dealt with a form that was primarily a free swimming one; in *Prorhynchus applanatus* with a form that was primarily a creeping one. *Prorhynchus stagnalis* usually creeps. It is, however, provided with mucous cells at the posterior end of its body by means of which it can attach itself to the substratum. From this stationary point it can move its body about in an almost complete circle, sometimes adhering closely to the surface, but frequently lifting the anterior third of its body, moving it either from side to side or backwards and forwards. Therefore, it would be natural to expect its special sense organs to be ventro-laterally disposed—and such happens to be the case. In *Prorhynchus stagnalis* is, therefore, presented a form that sometimes creeps and sometimes swims freely. So it is of interest to observe that when it is creeping the anterior end, through flattening, applies the ciliated pits to the substratum much as *Prorhynchus applanatus* does; whereas when the animal moves or sways through the water, its anterior end, through becoming rounded, places the ciliated pits more laterally as they are always placed in *Microstoma caudatum*. It has been thus observed that the position of the ciliated pits of *Prorhynchus stagnalis* also conforms with the diverse habits of *Prorhynchus stagnalis*.

The pits are directed obliquely posteriorly and mesially, there being a decided curvature in the "neck" of the pit.

In the histological structure of these pits we define three regions; the neck or transitory region (*t*), the sensory region (*s*), and the glandular region (*g*) (Figs. IV, V, and VII). All the components of the pits, with the single exception of the muscle fibers (Figs. IV, VI, and VII, *l.m.*, *m.m.*), which are attached to the pits, are considered to be of ectodermal origin. This assumption is based upon the fact that Kepner and Taliaferro ('12) in their study of the ciliated pits of *Microstoma caudatum*, clearly showed that in a newly-forming individual, both the glandular and sensory cells developed as modifications of an invagination of the general body epithelium or ectoderm (Figs. IV, V, and VII, *g.b.e.*). The pits of *Prorhynchus stagnalis* measure from twenty-five to thirty-five microns in length and from twelve to fifteen microns in width, (excluding the glandular cell, which is about two thirds of the length of the pit proper), being narrower near the mouth in the fixed condition on a slide than at the fundus of the pit. This latter condition, that of the narrowness of the mouth of the pit, is in sharp contrast to the contour of the functioning pit.

With the exception of the glandular region, the ciliated pit is, like the rest of the body epithelium, a syncytium, with its nuclei specialized to conform to their respective functions. The presence of a glandular cell makes even stronger the belief that the pit is a gustatory organ.

The nuclei of the ciliated, transitory epithelium are eight in number, a double row of four nuclei, one row being disposed obliquely dorso-ventrally, forming a part of the lateral wall of the pit, while the other row forms a similar mesial wall. These nuclei have an elongated, ovate contour with the axis of each nucleus in a plane that is at right angles to the axis of the pit, and bent to conform to the curved wall of the pit (Figs. IV, V, VI, and VII, *t*). They are densely granular, and the aspect of the possible fifth pair of nuclei nearest the mouth of the pit gradually fade into the appearance of nuclei of the general body epithelium.

There are also two rows of sensory nuclei, four in each row, and placed in the same plane as the nuclei of the transitory cells (Figs. IV, V, VI, and VII, *s*). On the inner walls of the pit next to the sensory nuclei are two rather densely granular ridges,

(Figs. IV, and VI, *r.*), presumably for the receiving of stimuli. These ridges are devoid of cilia, though the remaining inner surface of the pit is highly ciliated. Due to the difficulty in differentiating nervous tissue very clearly through any of the simpler staining methods, it was impossible to distinguish any special nerve endings. The main anterior nerves from the dorsal ganglia were closely applied to the outer surface of the pit along its mesial side only (Figs. IV, V, VI, and VII, *a.n.*). Until better stained sections can be studied, the matter of nerve application to the pit will have to rest with this general statement.

At the base of the lumen of the pit, and connected with it only through small pores (*p.*) which pass through the syncytial wall of the pit on the anterior angle of the fundus lies a large uni-cellular gland (Figs. IV, V, VI, and VII, *g.*). Its contour is very irregular, the cell giving off a number of processes. There are two interesting features to this glandular cell not noted in that of *Prorhynchus applanatus*. One concerns the characteristic bent, ovate contour of the nucleus of this cell (Figs. IV, V, and VII, *g.n.*), with clearly defined chromatin granules. The other interesting fact concerns the presence of a simple drainage system in the form of intra-cytoplasmic canaliculi (Figs. IV, V, VI, and VII, *cn.*), which pour the mucus-like secretions of the cell through the pores in the syncytial wall into the pit (Figs. IV, VI, and VII, *p.*). These canaliculi have been observed both in an empty state and filled with materials of secretion.

Connected with the ciliated pits are several non-striated muscle fibers, arising both laterally and mesially, and passing closely along the surface of the glandular cell. There is only one fiber attached to the lateral wall (Figs. IV and VII, *l.m.*), but at least three fibers attached to the mesial wall of the pit (Figs. VI and VII, *m.m.*).

Summary.

1. The correlation between the position of the ciliated pits in *Prorhynchus stagnalis* and the way in which the anterior portion of the body makes its exploratory movements is met in this rhabdocœle in the same manner as in *Microstoma caudatum* and *Prorhynchus applanatus*.

2. The ciliated pits of *Prorhynchus stagnalis* are composed of a

definite number of cells, seventeen each. In spite of the fact that *Prorhynchus stagnalis* has no specialized sensory tissue in the form of a pair of simple eyes, *Prorhynchus stagnalis* is considered being more highly developed in that a much larger number of cells go into the formation of the pits than in both of the sensory organs of *Prorhynchus applanatus*.

3. The ciliated pits of *Prorhynchus stagnalis* are sharply differentiated into three regions: transitory, sensory, and glandular.

4. There are two interesting features to the glandular cell not noted in *Prorhynchus applanatus:* (1), the characteristic curvature of the nucleus; and (2), the intra-cytoplasmic canaliculi.

5. The ciliated pits of *Prorhynchus stagnalis* possess a definite musculature, attached to both their mesial and lateral walls.

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EXPLANATION OF FIGURES.

PLATE I.

FIG. 1. Ventral aspect of Prorhynchus stagnalis to show especially the lateral expansion (*l.e.*) of the exploratory region anterior to the ciliated pits (*c.p.*). Chitinious penis (*ch.p.*); pharyngeal sac (*ph.s.*); pharynx (*ph.*); penis bulb (*p.b.*); enteron (*en.*); seminal vesicle (*s.v.*); vas deferens (*v.d.*); egg about to be liberated (*e.*); ovary (*ov.*); testes (*t.*); opening of female reproductive organs (♀); (Modified from Von Graff).

FIG. 2. Dorsal aspect of anterior third of body to show relation of ciliated pits (*c.p.*) to central nervous system. Pharyngeal sac (*ph.s.*); pharynx (*ph.*); dorsal commissure (*d.c.*); right dorsal ganglion (*d.g.*); right anterior nerve (*a.n.*); right posterior nerve (*p.n.*).

FIG. 3. Right lateral aspect of anterior third of body to show relation of ciliated pit (*c.p.*) to central nervous system. Pharyngeal sac (*ph.s.*); pharynx (*ph.*); dorsal commissure (*d.c.*); dorsal ganglion (*d.g.*); anterior nerve (*a.n.*); posterior nerve (*p.n.*).

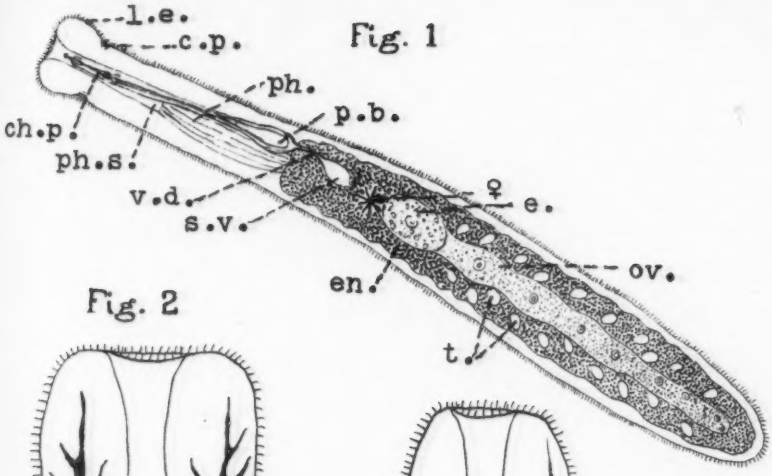

Fig. 1

Fig. 2

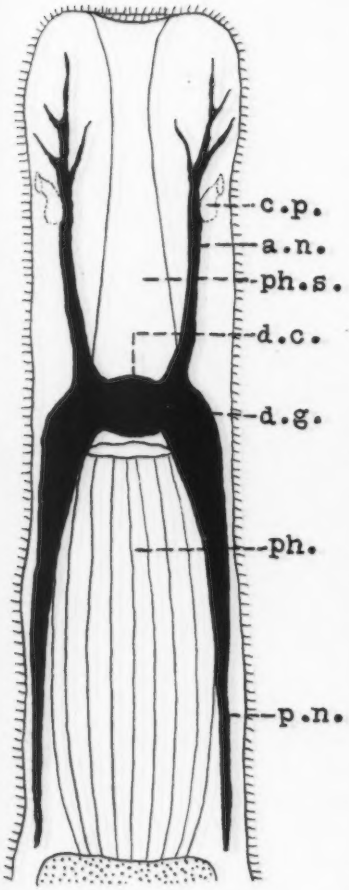

Fig. 3

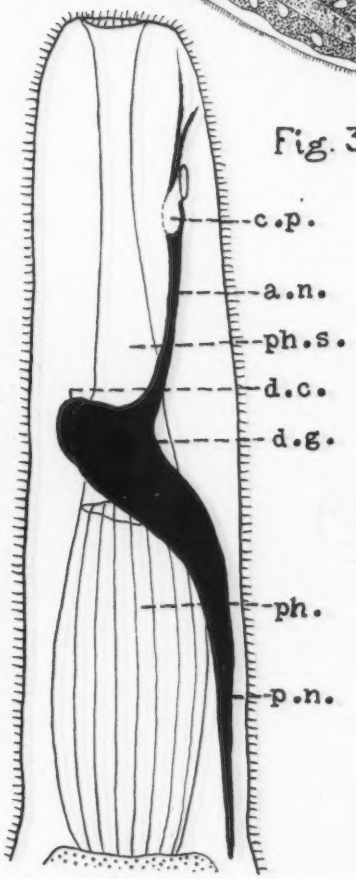

PLATE II.

FIG. 4. Longitudinal section through a left ciliated pit. General body epithelium (*g.b.e.*); transitory nucleus (*t.*); non-ciliated ridge (*r.*); sensory nucleus (*s.*); pore of gland cell (*p.*); canaliculi (*cn.*); gland cell (*g.*); anterior nerve (*a.n.*); nucleus of gland cell (*g.n.*); lateral muscle fiber (*l.m.*); × 1500.

FIG. 5. Longitudinal section through a left ciliated pit, especially to show the row of four sensory nuclei (*s.*); General body epithelium (*g.b.e.*); anterior nerve (*a.n.*); secretion in pores of gland cell which lead into lumen of pit (*se.*); canaliculi (*cn.*); gland cell (*g.*); nucleus of gland cell (*g.n.*); × 1500.

Fig. 5

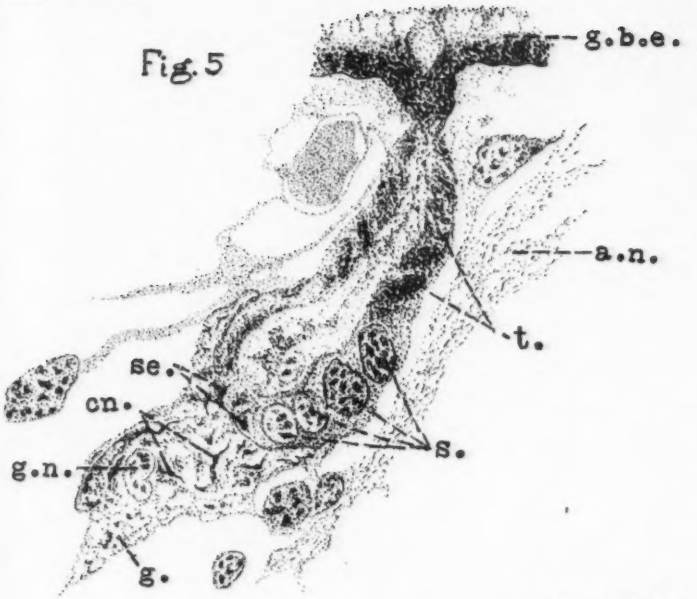

Fig. 4

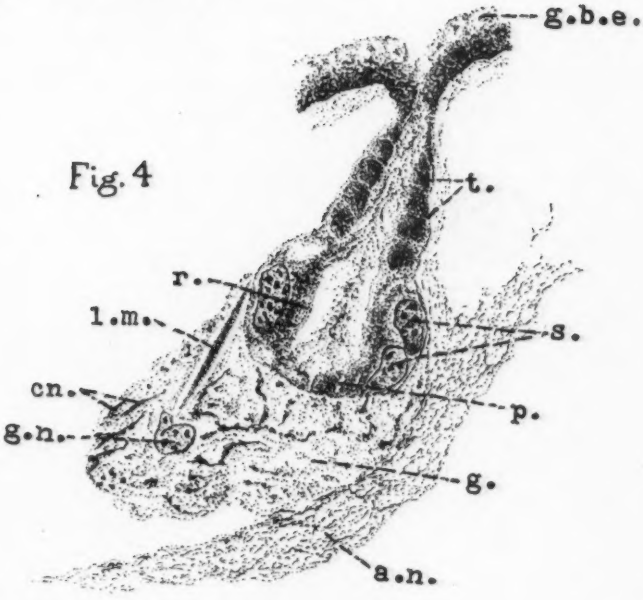

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PLATE III.

FIG. 6. Oblique-longitudinal section through right ciliated pit especially to show the three mesial muscle fibers (*m.m.*); associated with the ciliated pits. Sensory nucleus (*s.*); transitory nucleus (*t.*); pores of gland cell (*p.*); canaliculi (*cn.*); gland cell (*g.*); anterior nerve (*a.n.*); non-ciliated ridge (*r.*); × 1800.

FIG. 7. Diagrammatic reconstruction of lateral aspect of right pit. General body epithelium (*g.b.e.*); transitory nuclei (*t.*); mesial application of anterior nerve (*a.n.*); sensory nucleus (*s.*); pores of gland cell (*p.*); emptying into lumen of pit; canaliculi (*cn.*); gland cell (g.); bent, ovate nucleus of gland cell (*g.n.*); lateral muscles of wall of pit (*l.m.*); mesial muscles of wall of pit (*m.m.*); × 2000.

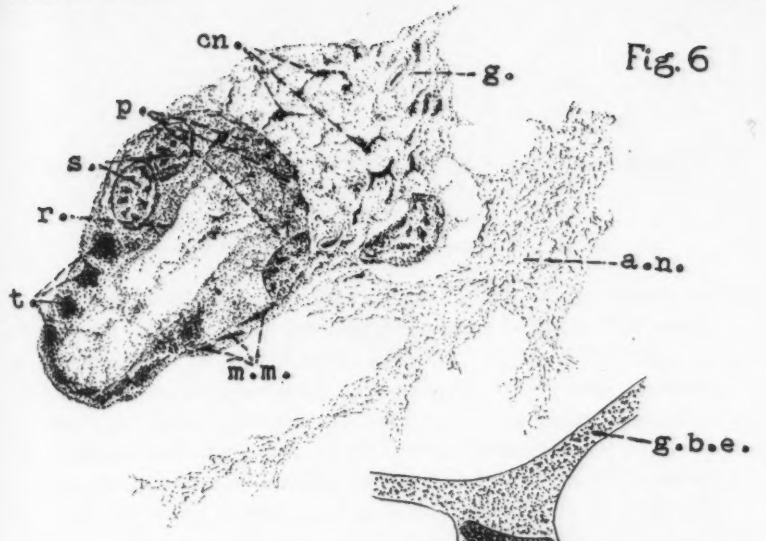

Fig. 6

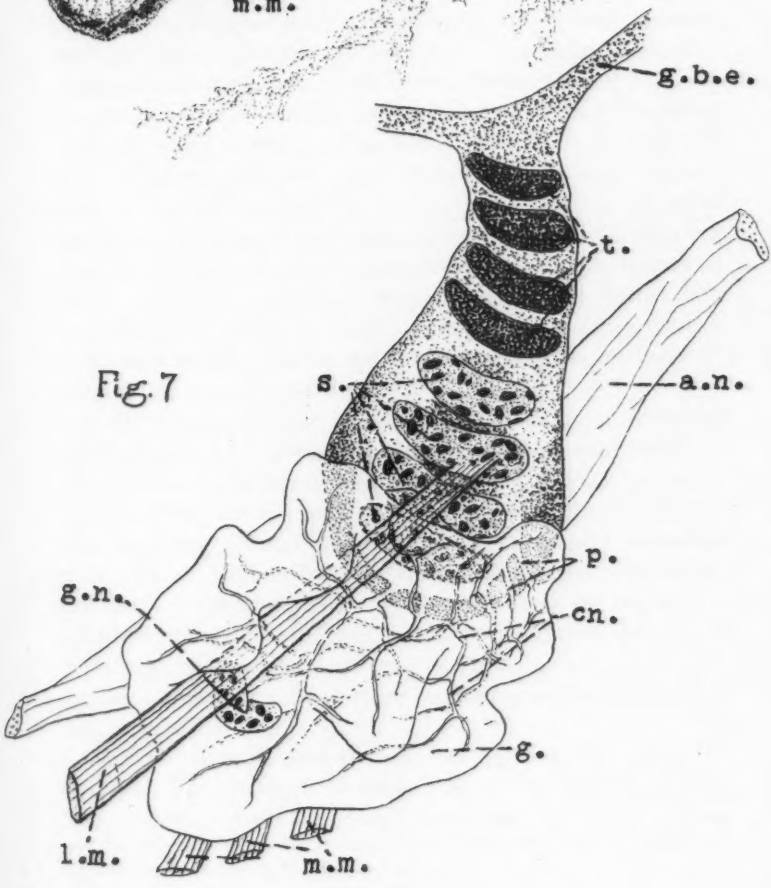

Fig. 7

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TESTICULAR ASYMMETRY AND SEX RATIO IN BIRDS.

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It has long been known that in some birds the right testis never attains the size of the left one even at the height of the vernal hypertrophy of the gonads. Newton, in his Dictionary of Birds (p. 784) writes that, ". . . generally the left testis is bigger than the right, although both are equally functional." Riddle,[1] working with pigeons at Cold Spring Harbor, found that the discrepancy in size between the two testes in hybrids increased with the width of the cross involved. That is to say, the left testis was proportionately larger in hybrid birds whose parents were of two different genera than in birds resulting from the mating of two congeneric species. The sex ratio of the offspring was found to be apparently interrelated with the discrepancy in size of the testes. Excess of males was always correlated with proportionately larger left testes, while in birds in which the two testes were equal in size the sex ratio was approximately one, *i.e.*, as many of one sex as of the other.

If there is anything in this seeming correlation it should be possible to arrive at some idea of the sex ratio in wild species of birds by examining the testes of adult males in breeding condition. Those in which the left testis is much larger than the right should have an excess of males in their total population and this excess should vary directly with the amount of difference between the two testes of the adult male. The importance of the sex ratio in such matters as the courtship habits and territorial relations of birds must be very great and its accurate determination for any species is prerequisite to a proper understanding of the habits of that species.

With the hope of getting some new light on this subject in wild birds I kept note of the relative size of the testes of all adult

[1] Riddle, Oscar, "Further Observations on the Relative Size of the Right and Left Testis of Pigeons in Health and Disease and as Influenced by Hybridity," *The Anatomical Record*, XIV., 1918, pp. 333–4.

male birds in breeding condition collected during three expeditions to South America, Africa, and the Texan-Mexican border. Only adult males in full breeding condition were of value in this study for in all the birds examined not in breeding condition the testes were invariably equal in size and very small.

In no species was the right testis larger than the left. The two were equal in size in one hundred and four species and the left testis was larger than the right (usually by a very considerable amount) in sixty species. Five species showed both conditions. This is hardly in keeping with Newton's statement quoted above. It should be borne in mind that many of the species are included in the following lists on the basis of the condition found in a single adult male. With further data and the elimination of individual variation the status of some of the species may require modification in this respect.

From my own field notes on all these birds and from all available published accounts of other observers it seems that no general correlation exists between the relative size of the testes of the breeding male and the sex ratio of the species. That is, field observations do not indicate a larger proportion of males in those species exhibiting testicular size assymetry than in those in which the two testes are equal. However it must be admitted that sex ratio is a very difficult thing to determine in the field, particularly as male birds are usually more conspicuous than females and are therefore seen and collected more frequently. In some small groups of species there does seem to be some such correlation as Riddle found in his pigeons. Perhaps the most striking case (and one on which I have sufficient material to eliminate individual variation) is that presented by the Cowbirds.

The Cowbirds are a group comprising three genera and six species. One[2] of these six species is extremely rare, being represented in the museums of the world by only one adult and three immature specimens and I have no data on it. The condition in the other five is interesting and suggestive. Two of them, *Agelaioides badius* and *Molothrus rufo-axillaris*, have no sexual dimorphism in plumage, are monogamous, and the ratio of the sexes is even, one male to one female. These two species have

[2] *Tangavius armenti.*

the testes equal in size. The other three *Molothrus bonariensis*, *Molothrus ater*, and *Tangavius æneus*, have sexual dimorphism in plumage, are more or less promiscuous with a tendency towards polyandry, and the males in all three outnumber the females by at least three to two, or by not less than fifty per cent. In these three species the left testis is much larger than the right. It is interesting to note that of the five the first two (with equal testes in breeding adult males) are the most primitive species of Cowbirds in all respects. *Agelaioides badius* is more or less normal in its reproductive habits but all the others are parasitic, *i.e.*, have no nests of their own and lay their eggs in nests of other birds and leave them to be cared for by these foster-parents. The development of this habit allowed for an increase of males in proportion to females as true mating and pairing were probably of less importance to a parasitic species than to one tied down by nesting and parental obligations. The parasitic habit is simplest in *Molothrus rufoaxillaris* and this species still pairs off in its ancestral monogamous fashion as does the most primitive, non-parasitic *Agelaioides badius*.

In most birds studied however no correlation between testicular asymmetry and sex ratio can be made out. Many more data are needed and I hope that bird collectors will make a point of noting the relative size of the testes in all breeding adult males.

BIRDS IN WHICH THE TWO TESTES ARE EQUAL IN BREEDING ADULT MALES.

Family Ciconiidæ.

Euxenura galatea (Molina).

Family Tinamidæ.

Nothura maculosa maculosa (Temminck).

Family Rallidæ.

Gallinula chloropus brachyptera (Brehm).

Family Charadriidæ.

Charadrius pecuarius pecuarius Temminck.

Family Jacanidæ.

Jacana jacana (Linneus).

Family Laridæ.

Sterna maxima Boddært.
Geochelidon nilotica (Gmelin).

Family Cuculidæ.

Crotophaga ani Linneus.
Guira guira (Gmelin).
Tapera nævia chochi (Vieillot).
Lampromorpha klaasi (Stephens).
Clamator cafer (Lichtenstein).
Coccyzus melocoryphus (Vieillot).
Coccyzus americanus americanus (Linneus).
Coccyzus erythrophthalmus (Wilson).

Family Coraciidæ.

Coracias caudatus caudatus Linneus.

Family Bucerotidæ.

Lophoceros erythrorhynchus erythrorhynchus (Temminck).

Family Trogonidæ.

Apaloderma narina narina (Stephens).

Family Trochilidæ.

Chlorostilbon aureoventris egregius Heine.
Heliomaster furcifer (Shaw).

Family Capitonidæ.

Trachyphonus erythrocephala erythrocephala Cabanis.

Family Indicatoridæ.

Indicator indicator Gmelin.
Indicator minor teitensis Neumann.

Family Picidæ.

Picumnus cirrhatus pilcomayensis Hargitt.
Campethera nubica pallida (Sharpe).
Trichopicus cactorum (d'Orbigny).

Family Furnariidæ.

Coryphistera alaudina alaudina Burmeister.
Phacellodomus rufifrons sincipitalis Cabanis.
Synallaxis superciliosa Cabanis.
Synallaxis albescens albescens Temminck.

Family Tyrannidæ.

Tænioptera irupero (Vieillot).
Fluvicola albiventer (Spix).
Euscarthmornis margaritaceiventer margaritaceiventer (d'Orbigny et Lafresnaye).
Elænia albiceps albiceps (d'Orbigny et Lafresnaye).
Empidagra suiriri (Vieillot).
Myiodynastes solitarius (Vieillot).
Pitangus sulphuratus bolivianus (Lafresnaye).
Tyrannus melancholicus melancholicus Vieillot.
Muscivora tyrannus (Linneus).

Family Alaudidæ.

Mirafra sabota Smith.
Mirafra africana transvaalensis (Hartert).
Eremopteryx leucopareia Fischer et Reichenow.

Family Motacillidæ.

Macronyx aurantiigula Reichenow.
Macronyx capensis (Linneus).
Anthus cafer (Sundeval).
Anthus leucophrys (Vieillot).

Family Timeliidæ.

Argya rubiginosa saturata Sharpe.

Family Pycnonotidæ.

Pycnonotus tricolor minor Heuglin.

Family Muscicapidæ.

Batis capensis (Linneus).
Alseonax adustus adustus (Boie).
Melænornis ater pammelaina (Stanley).
Elminia longicauda teresita Antinori.

Family Turdidæ.

Planesticus amaurochalinus (Cabanis).

Family Sylviidæ.

Erythropygia pæna pæna (A. Smith).
Sylvietta whytii loringi Mearns.
Cisticola semifasciata Reichenow.
Cisticola chiniana (A. Smith).
Polioptila cærulea cærulea (Linneus).
Polioptila dumicola (Vieillot).

Family Troglodytidæ.

Troglodytes musculus rex (Berlepsch et Leverkuhn).

Family Dicruridæ.

Dicrurus adsimilis adsimilis (Bechst.).

Family Laniidæ.

Eurocephalus rueppelli erlangeri Zedlitz.

Family Prionopidæ.

Prionops poliocephala talacoma A. Smith.

Family Vireonidæ.

Vireo chivi chivi (Vieillot).
Vireosylva olivacea (Linneus).

Family Paridæ.

Anthoscopus musculus (Hartlaub).

Family Oriolidæ.

Oriolus larvatus larvatus Lichtenstein.

Family Sturnidæ.

Amydrus morio morio (Linneus).
Spreo superbus (Ruppell).

Family Eulabetidæ.

Cinnyricinclus verreauxi (Finsch et Hartlaub).
Lamprocolius phœnicopterus bispecularis (Stickland).

Family Buphagidæ.

Buphagus erythrorhynchus (Stanley).

Family Zosteropidæ.

Zosterops virens Sundeval.

Family Nectariniidæ.

Nectarinia nectarinoides (Richmond).

Family Compsothlypidæ.

Dendroica æstiva æstiva (Gmelin).
Geothlypis æquinoctialis cucullata (Latham).
Setophaga ruticilla (Linneus).

Family Tanagridæ.

Thraupis bonariensis (Gmelin).

Family Ploceidæ.

Hyphantornis capensis olivaceus (Hahn).
Hyphantornis rubiginosus (Ruppell).
Hyphantornis nigriceps nigriceps Layard.
Hyphantornis jacksoni (Shelley).
Hyphantornis vitellinus uluensis Neumann.
Sycobrotus gregalis (Lichtenstein).
Euplectes xanthomelas Ruppell.
Pyromelana diadémata (Finsch et Reichenow).
Pytelia melba melba (Linneus).
Granatina granatina (Linneus).
Sporopipes squamifrons squamifrons (A. Smith).
Dinemellia dinemelli (Ruppell).
Philetairus socius socius (Latham).
Tetrænura regia (Linneus).

Family Bubalornithidæ.

Bubalornis niger intermedius (Cabanis).

Family Icteridæ.

Agelaioides badius badius (Vieillot).
Molothrus rufo-axillaris Cassin.
Icterus cucullatus sennetti Ridgway.

Family Fringillidæ.

Embernagra olivacens olivascens (d'Orbigny et Lafresnaye).
Saltator cærulescens cærulescens Vieillot.
Saltatricula multicolor (Burmeister).
Serinus mozambicus mozambicus (Linneus).
Sicalis pelzelni Sclater.
Sporophila lineola (Linneus).
Brachyspiza capensis argentina Todd.
Melospiza melodia melodia (Wilson).

BIRDS IN WHICH THE LEFT TESTIS IS LARGER THAN THE RIGHT.

Family Ardeidæ.

Butorides striatus cyanurus (Vieillot).

Family Palamedeidæ.

Chauna torquata (Oken).

Family Phasianidæ.

Francolinus coqui coqui (Smith).

Family Rallidæ.

Limnocorax flavirostra (Swainson).

Family Charadriidæ.

Charadrius collaris Vieillot.
Hoplopterus armatus (Burch.).

Family Laridæ.

Sterna superciliaris Vieillot.

Family Cuculidæ.

Cuculus clamosus jacksoni Sharpe.
Cuculus solitarius Stephens.
Clamator serratus (Sparrmann).
Chrysococcyx cupreus intermedius Hartlaub.
Centropus superciliosus furvus Friedmann.

Family Alcedinidæ.

Halcyon chelicuti chelicuti (Stanley).
Halcyon albiventris albiventris (Scop.).

Family Caprimulgidæ.

Setopagis parvulus (Gould).

Family Coliidæ.

Colius striatus minor Cabanis.

Family Indicatoridæ.

Prodotiscus regulus regulus Sundeval.

Family Phæniculidæ.

Phæniculus purpureus marwitzi (Reichenow).

Family Bucconidæ.

Nystalus maculatus striatipectus (Sclater).

Family Furnariidæ.

Furnarius rufus rufus (Gmelin).

Family Tyrannidæ.

Machetornis rixosa rixosa (Vieillot).
Pyrocephalus rubineus rubineus (Boddært).
Knipolegus aterrimus aterrimus Kaup.

Family Phytotomidæ.

Phytotoma rutila rutila Vieillot.

Family Alaudidæ.

Mirafra fischeri fischeri Reichenow.

Family Muscicapidæ.

Tchitrea viridis perspicillata Swainson.
Lioptilus nigricapillus (Vieillot).

Family Mimidæ.

Mimus saturninus modulator (Gould).

Family Campephagidæ.

Grauculus cæsia cæsia (Lichtenstein).

Family Laniidæ.

Chlorophoneus sulphureopectus suahelicus Neumann.
Laniarius funebris degener Hilgert.

Family Nectariniidæ.

Cinnyris mariquensis mariquensis Smith.
Cinnyris obscura neglecta Neumann.
Cinnyris chalybeus (Linneus).
Cinnyris amethystina amethystina (Shaw).

Family Ploceidæ.

Hyphantornis spilonotus (Vig.).
Melanopteryx nigerrima (Vieillot).
Nigrita schistacea Sharpe.
Anaplectes rubriceps (Sundeval).
Anomalospiza imberbis Cabanis.
Coliuspasser ardens (Boddært).
Coliuspasser eques Hartlaub.
Coliuspasser albonotatus (Cassin).
Diatropura procne (Boddært).
Urobrachya axillaris (A. Smith).
Pyromelana orix orix (Linneus).
Linura fischeri (Reichenow).

Family Icteridæ.

Agelaius phœniceus phœniceus (Linneus).
Molothrus ater ater (Boddært).
Molothrus ater obscurus (Gmelin).
Molothrus bonariensis bonariensis (Gmelin).
Tangavius æneus involucratus Lesson.
Quiscalus quiscula æneus (Ridgway).
Leistes superciliaris petilus Bangs.

Family Fringillidæ.

Passer domesticus (Linneus).
Passer melanurus (P. L. S. Muller).
Serinus canicollis thompsonæ Roberts.
Poliospiza gularis gularis (A. Smith).
Paroaria cristata (Boddært).
Emberiza flaviventris Stephens.

VARIABLE SPECIES.

Family Cuculidæ.

Cuculus clamosus clamosus Latham.
Lampromorpha caprius (Boddært).

Family Dendrocolaptidæ.

Lepidocolaptes angustirostris angustirostris (Vieillot).

Family Ploceidæ.

Vidua macroura (Vroeg.).
Hypochera orientalis Reichenow.

THE FUNCTION OF THE CONTRACTILE VACUOLE IN *PARAMECIUM CAUDATUM;* WITH SPECIAL REFERENCE TO THE EXCRETION OF NITROGENOUS COMPOUNDS.[1]

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INTRODUCTION.

Ehrenberg (1838) was probably the first to consider the function of the contractile vacuole. He asserted that it is a spermatic gland; but no evidence has been found in support of this view.

Lieberkühn (1836), Claparède (1834), Lachmann (1856), Siebold and Stannius (1854), and Pritchard (1861) held that it is a rudimentary heart, which, by its pulsations, produces the circulation of a body-fluid throughout the organism. The demonstration by Jennings (1904) that the vacuole communicates directly with the exterior and discharges its contents into the surrounding medium definitely eliminates such an explanation.

Haeckel (see Kent, 1880, p. 69), Maupas (1863), Bütschli (1887–89), Ehrmann (1894), and others contend that it is a respiratory organelle, or a mechanism for the removal of some of the end products of oxidation; but insufficient evidence has been found to warrant the acceptance of this view.

Stein and Schmidt (see Kent, 1880, p. 69), Griffiths (1888), Calkins (1909), Khainsky (1910), Woodruff (1911), Minchin (1912), Howland (1924), Nowikoff (1908), Shumway (1917), Riddle and Torrey (1923), Flather (1919), and Marshall (1921) believed the vacuole to be an excretory organelle. This view

[1] This paper is a portion of an essay submitted to the Board of University Studies of the Johns Hopkins University in partial fulfillment of the requirements for the degree of Master of Arts. The writer wishes to acknowledge his indebtedness to Professor S. O. Mast, who suggested the problem, and under whose personal supervision the work was done; and to Professor E. K. Marshall of the Department of Physiology of the Johns Hopkins Medical School who offered many helpful suggestions in connection with the literature on the enzyme, urease, and the xanthydrol test for urea.

is widely accepted, and is supported to some extent by experimental evidence. In certain instances, the term excretion is limited to mean only the expulsion of a fluid from the cell, as in Carter's (1861) observations. Generally, however, this theory assigns to the vacuole the function of a renal organ of some kind.

Hartog (1888), Calkins (1901), Zülzer (1910), Doflein (1911), and others maintain that the vacuole is an organelle for regulating the hydrostatic pressure within the cell, or a mechanism for removing the excess water which is taken into the body in feeding and through the cell membrane by osmosis. Stempell (1914) constructed a mechanical system which shows clearly that osmosis can be made the causal agent for producing intermittent discharge of a fluid from such a system, but this system was doubtless not intended to be compared with the vacuole in any respect other than the pulsating effect.

The literature concerned with the function of the contractile vacuole consequently reveals no conclusive evidence in support of one theory to the exclusion of all others. The consensus of the evidence, however, seems to indicate that the vacuole is an organelle concerned either with the removal of waste products of metabolism, or with the removal of excess water which accumulates in the organism as a result of endosmosis and feeding. The experiments described in the following pages have a direct bearing on the question as to which of these obtains. These experiments consist of attempts to ascertain the nature of the nitrogenous excretion products of metabolism in *Paramecium caudatum*, and whether or not they are excreted through the contractile vacuole.

The Nature of the Nitrogenous End Products in *Paramecium*.

The nitrogenous end products of metabolism in organisms vary in their nature according to the type of organism. For example, in man the bulk of the nitrogen is eliminated as urea, considerably less as uric acid and amino acids, and a very small part as free ammonia; while in the birds and reptiles the bulk of the nitrogen is eliminated as uric acid.

In connection with protozoa Griffiths (1888) made the statement, based on his own experiments, that the vacuole performs the function of a kidney, and that its secretions are "capable of yielding microscopic crystals of uric acid." As material for these experiments he used Amœba, Paramecium, and Vorticella, in mass cultures. In some of the experiments a number of amœbæ were placed on a slide and subjected to the murexide test. The development of reddish-purple color indicated the presence of uric acid. In describing these experiments Griffiths says (p. 132): "After the addition of alcohol minute flakes could be distinctly seen floating in the fluid of certain vacuoles. Bearing in mind the murexide reaction, there is every reason to believe that these flakes are nothing more or less than minute crystals of uric acid." These experiments were repeated many times, generally with positive results, indicating the presence of uric acid. At times, however, the vacuole was found not to contain the slightest trace of uric acid.

Howland (1924) repeated these experiments using several specimens of *Centropryxis* and *Amœba verrucosa*. Cells observed in a dark field immediately after the addition of alcohol did not show the crystals of uric acid in the distended vacuoles, nor did cells after the addition of ammonia show the characteristic murexide reactions, either in the vacuoles or in the culture medium immediately surrounding the organisms. These experiments were conducted during a period of five weeks, always with negative results. Paramecia were subjected to the same test, also with negative results. Howland made use of the Benedict blood-filtrate test for uric acid on cultures of paramecia and amœbæ with positive results. The depth of the color developed varied with the age of the cultures, the older ones giving a deeper color. This indicates that uric acid was eliminated in some way by the organisms.

The question then arises: Is nitrogen excreted by protozoa as ammonia, urea, uric acid, or a combination of these substances? In an effort to answer this question the following experiment was conducted. A large number of paramecia were thoroughly washed, placed in spring water that was free from ammonia and urea, left for a time, and removed by filtration. The

filtrate was then tested for ammonia and urea as described below. The possible presence of uric acid was not investigated at this time.

The paramecia were washed as follows: Culture fluid containing the organisms was poured into a long-necked bottle of approximately one liter capacity until it was filled to within about 5 cm. of the top. Then spring-water was added carefully so as to avoid mixing until the bottle was entirely full. Within five or ten minutes under these conditions the paramecia usually aggregated in very great numbers near the surface of the spring-water. When they had thus collected at the top of the bottle, the surface water containing them was removed with a pipette, and more spring-water added. This process was repeated until most of the animals had been taken from the culture fluid, usually three or four times. The bottle was then emptied, and the water containing the paramecia put into it, after which it was filled with spring-water. The organisms were removed as before. The paramecia were thus washed in fresh spring-water three or four times, after which they were usually found to be free from all heavy debris and large bacterial masses. Smaller organisms which were removed from the bottle with the paramecia were separated from the paramecia by further washing on filter paper. The paper used was about 20 cm. in diameter, and was selected so that the pores were small enough to retain the paramecia, but large enough to allow the smaller organisms to pass through. A liter of water, or more if necessary, was used for this part of the washing process.

After the paramecia had been thus washed they were put into a clean glass beaker; then spring-water was added until the number per cubic centimeter was reduced to from 500 to 2,500 individuals. This was ascertained by counting the numbers in several one cubic centimeter portions and averaging the results obtained. The paramecia were allowed to remain in this water for periods of time ranging from eighteen to thirty-six hours, after which they were removed by filtration.

A portion of the filtrate was tested for ammonia by Nesslerization and the rest for urea by methods described below.

In twenty-two of the twenty-five experiments positive tests

were obtained for ammonia by Nesslerization. In the three in which no ammonia was found the paramecia had been in the water for a period of thirty hours or less. In other experiments in which the length of time was thirty-six hours or more, ammonia was invariably found. This indicates that either ammonia was eliminated in such small amounts that more than thirty hours were required for the concentration to rise sufficiently high to be detected by Nesslerization; or that no ammonia was eliminated as such, the positive test being due to that formed from the hydrolysis of some other excretion product. The latter seems the more probable, for, if ammonia was excreted, the length of time necessary for its concentration to rise sufficiently high to be detected should bear an inverse relation to the number of paramecia per unit volume of water. No relation of this kind was found to exist. The ammonia appeared after thirty to thirty-six hours in all the experiments regardless of the number of animals present. The maximum variation, then, in the length of time necessary for ammonia to make its appearance was twenty per cent., while the variation in the number of paramecia in these same experiments was one hundred per cent. or more. The absence of ammonia in three experiments, and its presence in all the others can be explained if it is assumed that there were too few bacteria present in the three to produce hydrolysis of the more complex excretion products, while in the other experiments there were enough bacteria present. That ammonia can be produced in this way was demonstrated by inoculating a dilute solution of urea with culture fluid. After the solution had been allowed to stand for several hours it gave a positive test for ammonia with Nessler's reagent, indicating that hydrolysis had taken place.

Many tests were made of the materials used in these experiments to prevent the possible introduction of errors. The spring-water was tested for ammonia. The sensitivity of Nessler's reagent was ascertained by finding the greatest dilution possible at which a definite indication of ammonia could be obtained. This dilution was found to be approximately one part in two million. The filter paper on which the paramecia were washed was tested for ammonia.

To ascertain whether or not ammonia is present in the fluid of the vacuole, fifteen experiments were conducted in which Nessler's reagent was injected into the organism. The apparatus used in making these injections consisted of the micropipette developed by Taylor (1925) mounted on the micromanipulator developed by Chambers (1922). The process of injection was performed with the paramecium held by surface tension in a hanging drop of water. The cover-glass bearing the organism formed the top of a cell, the front of which was left open to allow the micropipette entrance. The tip of the pipette was bent up at a right angle to the main shaft to facilitate the injection of the organism suspended on the lower surface of the cover-glass.

In twelve of the injections the contents of the pipette were discharged into the vacuole. In three the pipette did not penetrate the vacuole, but discharged its contents into the cytoplasm in the immediate vicinity of the vacuole. In every test the reagent, which is highly caustic, caused the immediate solution of the whole organism with the exception of the nucleus, which remained intact for a short time before it too was dissolved. In the three tests in which the pipette did not penetrate the vacuole, the surrounding cytoplasm was dissolved as before, but the membrane around the vacuole remained intact for a short time. After several seconds the membrane was dissolved, causing the contents of the vacuole to be emptied into the solution of Nessler's reagent in which it was floating. In none of these tests was the characteristic straw color observed which, in the presence of Nessler's reagent, indicates ammonia. It seems, then, that if ammonia is present in any part of the organism its concentration is below the sensitivity of the reagent. All of these experiments seem to indicate then that very little if any of the nitrogen found in the excretion products of *Paramecium* is excreted in the form of ammonia.

The test for urea referred to above was made as follows: Urease, a specific enzyme for urea, hydrolyzing it into ammonia and carbon dioxide, was added to the portion of the filtrate not used for the test for ammonia in each of the twenty-five experiments mentioned. They were then left for several hours, after

which they were tested for ammonia by Nesslerization. Ammonia was found in the filtrate from every experiment, and generally in higher concentrations than it was in the portions to which no urease had been added. This increased ammonia content after hydrolysis may, then, with a reasonable degree of certainty, be interpreted as indicating that urea from some source had been hydrolyzed with the subsequent production of ammonia. The fact that the three filtrates which gave no indication of ammonia before hydrolysis, gave a distinctly positive test for ammonia after hydrolysis, is alone conclusive in so far as the action of urease is known to be limited to the hydrolysis of urea. Since paramecia were the only organisms present in the water in any considerable numbers, the source of this urea must be attributed to them. It therefore seems evident that in *Paramecium* at least some of the nitrogen is excreted in the form of urea.

Is Urea Eliminated by the Contractile Vacuole?

In an effort to answer this question the xanthydrol precipitation test for urea, described by Fosse (1913) and modified to suit conditions of this experiment, was made by injecting the reagent into the vacuoles. The modified reagent consisted of three to five drops of a ten per cent. solution of xanthydrol in methyl alcohol, in 1 cc. glacial acetic acid. This reagent, in the presence of urea, precipitates long, needle-like crystals of di-xanthyl urea which may be easily recognized. The sensitivity of the modified reagent was found by injecting it, with the aid of the apparatus described above, into a drop of a solution of urea of known concentration. The solution of urea used was successively diluted until the urea content was so low that no precipitate could be observed. It was found that one or more parts of urea in twelve thousand could be detected. The process of injecting the reagent into the drop of solution was observed under a microscope.

Considerable annoyance was encountered in attempting to inject the contractile vacuole of *Paramecium* with the xanthydrol reagent in that fumes from the acetic acid in the pipette killed the organism before the injection could be made. This difficulty

was finally overcome by drawing into the pipette a very small amount of paraffine oil after the pipette had been filled with the reagent. The oil is chemically inert under ordinary conditions and served the purpose very well.

The effect of the reagent on the five paramecia successfully injected was quite striking. The animal was fixed immediately. It assumed an almost hyaline appearance with the exception of the nucleus, food granules, and numerous short, thick crystals which are normally found throughout the body. The contractile vacuole disappeared completely. No trace of the characteristic needle-like crystals of di-xanthyl urea, which are precipitated by xanthydrol in the presence of urea, were found either in that part of the organism in which the vacuole is usually situated, or in the liquid surrounding the organism. Some of these observations were made under an apochromatic oil immersion lens system. It seems from this, then, that if urea is present in the fluid of the vacuole its concentration is too low to be detected with the reagent used, that is, one part in 12,000.

Now the question arises as to whether or not all of the urea excreted could be eliminated by the contractile vacuole if the concentration is as low as this. If not, then it is evident that the contractile vacuole does not function specifically in the excretion of nitrogen, and if this is true it is not an excretory organelle in the ordinary sense of the term.

The concentration of urea that should be in the fluid of the vacuole, if all of it is eliminated through it, was ascertained in the following manner. Maupas (1883) found that the vacuoles of *Paramecium aurelia* evacuate a quantity of water equal to the volume of the entire organism in forty-six minutes at twenty-seven degrees. It was assumed in making these calculations that the relative quantity of water evacuated by *Paramecium caudatum* is approximately equal to that evacuated by *Paramecium aurelia* during the same period and at the same temperature. The average volume of *Paramecium caudatum* was assumed to be that of a cylinder 150 microns long and 35 microns in diameter, and the diameter of the vacuole when distended 10 microns.

On the basis of these assumptions and the observations of

Maupas, the volume of water evacuated by a definite number of paramecia in a definite period of time was calculated. From this the concentration of urea that would be in the fluid of the vacuole, if its function is excretory, was computed. The results of these calculations show that the concentration of urea in the fluid of the vacuole would have to be of the order of one part in two or three thousand to eliminate through it the calculated amount of urea.

The reagent injected into the vacuole is, as previously stated, sensitive to one part of urea in twelve thousand. Since there was in these injections no indication of the presence of urea, it is evident that the results of these experiments are in opposition to the theory that the vacuole is an organelle whose function is the removal of the nitrogenous waste products of metabolism, unless it functions specifically in eliminating uric acid, which is not probable. If, then, it is true that the contractile vacuole functions either as an excretory organelle or a mechanism for regulating the hydrostatic pressure within the cell, it is evident that the results support the latter.

Summary

1. The presence of ammonia and urea in *Paramecium* cultures has been demonstrated.

2. Ammonia is due to the hydrolysis of urea, and is not excreted as such.

3. Nitrogen is eliminated in the form of urea.

4. All the urea excreted can not be eliminated through the vacuole.

5. The function of the vacuole is not the elimination of nitrogenous waste products of metabolism, but is probably the regulation of the hydrostatic pressure within the cell.

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THE USE OF NILE BLUE SULFATE AS A VITAL STAIN ON *HYDRA*.

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In connection with some studies on the reconstitution of *Hydra*, it occurred to the writer that if some method could be devised for marking the various regions in an animal and if these markings would persist, the process of reconstitution could be followed and interpreted much more easily and accurately. However, in reviewing the literature there seems to be little or no application of any of the so-called vital dyes to the invertebrates, particularly the Cœlenterata.

Various investigators in studying regeneration in *Hydra* following grafting have made use of animals of two different species which differed from each other, among other characteristics, in color. In this way the fate of the graft could be traced. Others have used individuals of the same species which differed from each other in color shade. Both of these procedures are open to criticism in interpretation of results. In the case first mentioned there are undoubtedly present species differences other than color between the animals used, such as differences in the metabolic rate of the animal as an individual and differences in gradient patterns. In the second case in individuals of the same species, color differences seem to be indicative of different physiological conditions. Observations tend to show, for example, that young *Pelmatohydra oligactis* (Schulze) Pallas (*Hydra fusca* L.) are lighter in color than old animals. The same color difference seems to be true of starved animals as compared to fed. From data gathered by various investigators on *Tubularia* and *Planaria*, there are marked physiological differences between young and old, and starved and fed animals with respect to metabolic rate and, consequently, capacity for reconstitution under various conditions. Obviously some method of vital staining would remove the necessity for using such methods of mark-

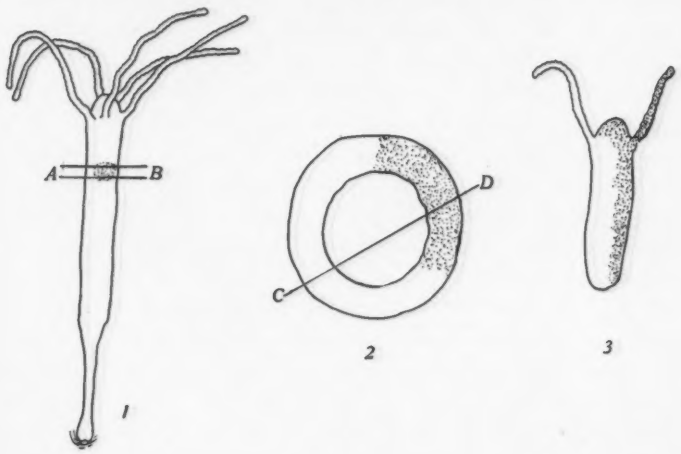
ing as previously described and permit experimentation under more normal, controlled conditions.

Of the various stains considered, nile blue sulfate seemed the most suitable for the work on *Hydra*, particularly on *Pelmatohydra* which is brown in color. The use of nile blue sulfate as a vital stain has been confined for the most part to embryological investigations and applied here for marking the eggs or the embryos. Goodale ('17) used nile blue sulfate in marking the eggs of *Spelerpes bilineatus*, the dye being applied locally in solid form in the least possible amounts by means of a needle. The dye particle was left for a few moments and then removed by washing. A too prolonged application or the application of the dye in excess amount was found to be toxic. Detweiler ('17) used the dye in aqueous solutions of 1 : 100,000 up to 1 : 500,000 but concluded that the ratio of 1 : 150,000 was optimum. In this case the entire animal was stained. Smith ('14) applied an aqueous solution by means of a fine pipette and was able to get some blue spots which persisted. Vogt ('25) uses nile blue sulfate in vital staining by first staining finely divided agar in aqueous solution of the dye (conc. 1 : 100 to 1 : 1,000) and after a few days applying in water one of the colored pieces of agar to the tissue to be stained. The color diffuses over after an interval of several hours to one day. However, the stain is not well localized. To localize the dye by this method, the stained agar must be inclosed in glass, tinfoil or paraffin.

After various trials, the method which was found most successful was that of Goodale. The animal, *Pelmatohydra oligactis* (Schulze) Pallas (*Hydra fusca* L.), was placed on a glass slide and the excess water removed from around the animal by means of filter paper. The animal was then transferred on the slide to a dissecting microscope and the smallest possible particle of nile blue sulfate applied in the desired region by means of a needle. Almost immediately the animal was washed from the slide into a stender dish containing water and thoroughly washed in currents of water by means of a pipette. A blue spot was found on the animal at the place of application of the dye. The animal was then sectioned in any way desired and in the following

process of reconstitution of the pieces the blue color was found confined to the original colored cells or the new cells derived from this group as the following example will serve to illustrate.

An animal was stained as described above and a piece removed by two parallel cuts, transverse to the longitudinal axis of the animal (Fig. 1, *ab*). This cross-section, when removed from the animal, appeared as a ring of tissue with the blue spot (stippled area) at one point in the circumference (Fig. 2). This ring of tissue was then divided into two equal portions by a cut (Fig. 2, *cd*) which passed through the blue spot. The two pieces were allowed to reconstitute and at the end of four days two small animals were found similar in marking to Fig. 3.



The process of reconstitution was as follows: the two cut lateral ends of each semicircle had approached and fused, thus forming two small rings similar to that seen in Fig. 2. Further process of reconstitution had then ensued governed by the original polarity of the pieces as is indicated by the color pattern as seen in Fig. 3. Further application of this staining method has been made in investigations not published as yet.

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